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(54) Title: METHODS AND COMPOSITIONS RELATING TO BODY WEIGHT AND EATING DISORDERS

(57) Abstract: Methods and compositions relating to body weight and eating disorders, such as obesity, are disclosed. Specifically, proteins that are differentially expressed in these conditions are identified. In one aspect, the invention provides a method of screening an agent to determine its usefulness in treating a condition associated with regulation of appetite and body weight, the method comprising: (a) establishing a paradigm in which at least one protein is differentially expressed in relevant tissue from, or representative of, subjects having differential levels of body weight or eating dysfunction; (b) obtaining a sample of relevant tissue taken from, or representative of, a subject having body weight or eating disorders, who or which has been treated with the agent being screened; (c) determining the presence, absence or degree or expression of the differentially expressed protein or proteins in the tissue from, or representative of, the treated subjects; and (d) selecting or rejecting the agent according to the extent to which it changes the expression of the differentially expressed protein or proteins in the treated subject having body weight or eating disorders.

Methods and Compositions Relating to Body Weight and Eating Disorders

Field of the Invention

5   The present invention relates to methods and compositions
       relating to body weight disorders and eating disorders in
       conditions such as obesity. Specifically, the present
       invention identifies and describes proteins that are
       differentially expressed in body weight and/or eating

10   disordered states relative to their expression in normal
       or non-body weight disordered states and in particular
       identifies and describes proteins associated with the
       regulation of appetite and body weight. Further, the
       present invention identifies and describes proteins via

15   their ability to interact with gene products involved in
       the regulation of body weight and appetite. Still
       further, the present invention provides methods,
       particularly experimental paradigms for the
       identification of differentially expressed proteins that

20   are potential molecular targets for compounds to treat
       body weight and/or eating disorders including, but not
       limited to, obesity. Still further, the present
       invention provides methods for the identification and
       therapeutic use of compounds for the treatment of body

25   weight and/or eating disorders including, but not limited
       to, obesity.

Background of the Invention

       Body weight disorders, including eating disorders, are
30   one of the major public health problems in all
       industrialised countries and is a growing problem in
       countries undergoing rapid acculturalisation. Obesity,
       the most prevalent of eating disorders, is the most
       common nutritional disorder in the Western world and can

have a prevalence of up to 50% in middle-aged and elderly populations. However, it is also an increasing problem in children. Other weight disorders, such as anorexia nervosa and bulimia nervosa are also serious health risks

5   and affect approximately 0.2% of the population, particularly female, in Western countries. Anorexia and cachexia are major features of other diseases such as cancer, AIDS and trauma.

10   Obesity, defined as an excess body fat relative to lean body mass, also contributes to many other diseases. For example, obesity is responsible for the increased incidence of diseases such as coronary artery disease, stroke and non-insulin dependent diabetes. Obesity is

15   not merely a behavioural problem, i.e. the result of voluntary hyperphagia. Rather, the differential body composition observed between obese and non-obese subjects results from differences in both metabolism and neurological/metabolic interactions. These differences

20   are to some extent genetically inherited but the nature of the gene products that control body weight and body composition are unknown. Attempts to identify protein molecules involved in the control of body weight have been largely empiric and the nature of the mechanisms by

25   which body composition and/or substrate flux are monitored have not yet been identified.

The epidemiology of obesity strongly shows that the disorder exhibits inherited characteristics (Stunkard,

30   1990, N. Eng. J. Med. 322: 1483). Moll et al., have reported that, in many populations, obesity seems to be controlled by a few genetic loci (Moll et al., 1991, Am. J. Hum. Gen. 49: 1243). In addition human twin studies

strongly suggest a substantial genetic basis in the
control of body weight, with estimates of heritability of
80-90% (Simopoulos, A.P. & Childs, B., eds., 1989, in
"Genetic Variation and Nutrition in Obesity", World
5   Review of Nutrition and Diabetes 63, S. Karger, Basel,
Switzerland; Borjeson, M., 1976, Acta. Paediatr. Scand.,
65:   279-287).

Further, studies of non-obese persons who deliberately
10   attempted to gain weight by systematically over-eating
found that some were more resistant to such weight gain
than others and were able to maintain an elevated weight
only by very high caloric intake. In contrast,
spontaneously obese individuals are able to maintain
15   their obese status with normal or only moderately
elevated caloric intake.

In addition, it is a commonplace experience in animal
husbandry that different strains of swine, cattle, etc.,
20   have different predispositions to obesity. Studies of
the genetics of human obesity and of models of animal
obesity demonstrate that obesity results from complex
defective regulation of both food intake, food induced
energy expenditure and of the balance between lipid and
25   lean body anabolism.

There are a number of genetic diseases in man and other
species, which feature obesity among their more prominent
symptoms, along with, frequently, dysmorphic features and
30   mental retardation. Although no mammalian gene
associated with an obesity syndrome has yet been
characterised in molecular terms, a number of such
diseases exist in humans. For example, Prader-Willi

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syndrome (PWS) affects approximately 1 in 20,000 live
births, and involves poor neonatal muscle tone, facial
and genital deformities, and generally obesity. The
genetics of PWS are very complex, involving, for example,
5   genetic imprinting, in which development of the disease
seems to depend upon which parent contributes the
abnormal PWS allele. In approximately half of all PWS
patients, however, a deletion on the long arm of
chromosome 11 is visible, making the imprinting aspect of
10   the disease difficult to reconcile. Given the various
symptoms generated, it seems likely that the PWS gene
product may be required to normal brain function, and
may, therefore, not be directly involved in adipose
tissue metabolism.

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In addition to PWS, many other pleiotropic syndromes,
which include obesity as a symptom, have been
characterised. These syndromes are more genetically
straightforward, and appear to involve autosomal
20   recessive alleles. The diseases, which include, among
others, Ahlstroem, Carpenter, Bardet-Biedl, Cohen, and
Morgagni-Stewart-Monel Syndromes. However, each of these
is rare and they do not account for the human obesity
epidemic.

25

There are a number of animal models with mutations that
are associated with body weight and body composition
disorders and attempts have been made to utilise such
animals as models for the study of obesity. The best
30   studied animal models for genetic obesity are mice, which
contain the autosomal recessive mutations ob/ob (obese)
or db/db (diabetes). These mutations are on chromosome 6
and four respectively, but lead to clinically similar

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pictures provided the genes are expressed on the same
background strain. The ob gene product has been
identified as 16kDa polypeptide produced primarily by
adipose tissue that provides a signal to the brain on the
adipose tissue fat stores. Mice with a mutation,
resulting in no circulating protein (called leptin) are
hyperphagic, obese, have poor thermo-regulation and non-
shivering thermogenesis and are insulin resistant with
impaired glucose tolerance. Treatment of these mice with
recombinant leptin reduces food intake and stimulates
energy expenditure so that the mice become less obese.

The db/db mice have a mutation in the receptor for leptin
so that normal signal transduction via the JAK/STAT
pathway does not occur. This mutation, when on the
C57BI/6 background, is phenotypically identical to the ob
mutation, but causes additionally frank diabetes when on
the C57BI/Ks background. Other single gene mutations in
mice associated with obesity, include the yellow mutation
at the agouti locus, mutations at the fat and tubby loci
and an autosomal dominant mutation at the adipose locus
on chromosome 7.

Other mutant animal models include fa/fa (fatty) rats and
ZDF fatty rats, which bear strong respective similarities
with the ob/ob and db/db mice. Thus the fa/fa rat is
hyperphagic, obese, insulin resistant, very
hyperinsulinaemic and glucose intolerant, whereas the ZDF
rat is hyperphagic, obese, insulin resistant and
hyperinsulinaemic, but develops frank diabetes after
approximately 6 weeks of age.

Inbred mouse strains, such as the NZO mouse, the Japanese

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KK mouse are models of obesity. Further, desert rodents, such as spiny mice and sand rats are neither insulin resistant nor diabetic in their natural habitats, but do become insulin resistant and glucose intolerant when fed on a standard laboratory diet.

Obesity is a common feature of elderly rodents and the development of obesity can be accelerated by feeding diets with a high fat content, whether these diets are synthetic homogenous diets or are the result of supplementation of replacement of the normal rat chow by human food with a high fat content (cafeteria diet).

In animal studies, it has been demonstrated that, as in humans, some animals are able to resist the obesity inducing effects of a diet with a high fat content. This resistance appears to be at, at least, two levels. First, there are differences in preferences for diet high in fat. Thus, some animals do not overeat when presented with a high fat diet, whereas others do. Secondly, animals show differences in their ability to resist obesity by activating energy wasting mechanisms.

Farm animal studies have clearly demonstrated that selective breeding can be undertaken for common traits such as food efficiency, growth rate, leanness. Similarly studies in mice have been undertaken to selectively breed for obesity and leanness so that over several generations of brother/sister matings colonies of mice are produced from a common parent stock that are significantly different in the level of fatness.

All of these animal models have been used from time to

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time to evaluate new drugs that were potential treatments
for body weight and eating disorders, including obesity.
However, although individual changes in enzyme activities
have been identified in some of the animal models and how

5   this might be altered by a drug therapy, no systematic
evaluation has been made of the differences in protein
expression in the tissues of normal animals and the obese
animals. It is these changes in protein expression that
underlie the development of body weight and eating

10   disorders, including obesity. It is the same changes in
protein expression that are likely to be causative of
obesity in humans and in companion animals such as dogs
and cats. Given the severity and prevalence of obesity,
there exists a great need for the systematic

15   identification of the disease causing proteins, since
modulation of the expression level of such proteins back
to the level in non-obese state represents a means of
treating the disease condition.

20   **Summary of the Invention**
Broadly, the present invention relates to methods and
compositions for the treatment of body weight and eating
disorders, including but not limited to, obesity. More
specifically, the present invention identifies and

25   describes proteins that are differentially expressed in
body weight and/or eating disordered states relative to
their expression in normal, or non-body weight disordered
states and also identifies proteins that are
differentially expressed in response to manipulations

30   relevant to body weight regulation and/or control of food
intake. Such differentially expressed proteins may
represent 'target proteins' and/or fingerprint proteins.
Further, the present invention identifies and describes

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proteins termed pathway proteins via their ability to
interact with proteins involved in the regulation of body
weight and/or food intake. Pathway proteins may also
exhibit target protein and/or fingerprint protein
5   characteristics.

Accordingly, in the first aspect, the present invention
provides a method of screening an agent to determine its
usefulness in treating a condition characterised by body
10   weight disorders and/or eating disorders, the method
comprising:
       (a)   establishing a paradigm in which at least one
protein is differentially expressed in relevant tissue
from, or representative of, subjects have differential
15   levels of obesity;
       (b)   obtaining a sample of relevant tissue taken
from, or representative of, a subject have obesity, who
or which has been treated with the agent being screened;
       (c)   determining the presence, absence or degree of
20   expression of the differentially expressed protein or
proteins in the tissue from, or representative of, the
treated subject; and,
       (d)   selecting or rejecting the agent according to
the extent to which it changes the expression of the
25   differentially expressed protein or proteins in the
treated obese subject.

Typically, an agent is selected if it changes the
expression of a differentially expressed protein towards
30   that of a normal weight subject.

In a further aspect, the present invention provides a
method for the identification of an agent or agents for

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use in the treatment of body weight and/or eating
disorders comprising the steps of:

(a)   identification of experimental and/or clinical
paradigms that exhibit differential levels of obesity or
lean or nutrient intake status (e.g. fed v fasted) or
macronutrient selection (e.g. fat-preferring v non-fat
preferring);

(b)   identification of differentially expressed
proteins in tissues of animals or humans exhibiting
differential levels of obesity or leanness or nutrient
intake states or macronutrient selection;

(c)   selecting an agent that converts the expression
of differentially expressed proteins in tissues of
animals or humans exhibiting body weight or eating
disorder dysfunctional states to that in the normal state
for use in the treatment of the body weight or eating
disordered state.

In a further aspect, the present invention provides the
use of an agent identified by the above method for the
preparation of a medicament for the treatment of a
condition characterised by body weight or eating
disorders. These conditions include obesity, non-insulin
dependent diabetes (type 2 diabetes), Cushing syndrome,
anorexia nervosa and bulimia.

In a further aspect, the present invention provides a
method of treating a condition characterised by body
weight or eating dysfunction in a patient, the method
comprising administering a therapeutically or
prophylactically effective amount of such an agent
identified by the above method.

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In a further aspect, the present invention provides a
method of determining the nature or degree of body weight
and/or eating dysfunction in a human or animal subject,
the method comprising:

5   (a)   establishing a paradigm in which at least one
protein is differentially expressed in relevant tissue
from, or representative of, subjects having a body weight
and/or eating disorder;

           (b)   obtaining a sample of the tissue from the
10   subject;

           (c)   determining the presence, absence or degree of
expression of the differentially expressed protein or
proteins in the sample; and

           (d)   relating the determination to the nature or
15   degree of the body weight or eating disorder by reference
to a previous correlation between such a determination
and clinical information.

Conveniently, the patient sample used in method can be a
20   tissue sample or body fluid sample or urine. This method
allows the causes of body weight or eating disorder
dysfunction of a patient to be correlated to different
types to prophylactic or therapeutic treatment available
in the art, thereby enhancing the likelihood of obtaining
25   a beneficial response in the patient to the therapy.

In a further aspect, the present invention provides a
method of treatment by the use of an agent that will
restore the expression of one or more differentially
30   expressed proteins in the body weight or eating disorders
dysfunction state to that found in the normal state in
order to maintain a reduced body weight (prevent weight
gain) or to prevent the re-emergence of an eating

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disorder.

In a further aspect, the present invention provides a
method whereby the pattern or differentially expressed
5   proteins in a tissue sample or body fluid sample or urine
of an individual with body weight and/or eating disorder
dysfunction is used to predict the most appropriate and
effective therapy to alleviate the dysfunction state and
to monitor the success of that treatment.
10

**In a further aspect, the present invention provides a
protein which is differentially expressed in relevant
tissue from, or representative of subjects having
differential levels of obesity or leanness or nutrient
15   intake states or macronutrient selection and which is as
obtainable by the method of two-dimensional gel
electrophoresis carried out on said tissue or a protein-
containing extract thereof, the method comprising:**
         (a)   providing non-linear immobilised pH gradient
20   (IPG) strips of acrylamide polymer 3mm x 180mm;
         (b)   rehydrating the IPG strips in a cassette
containing 25ml of an aqueous solution of urea (8M), 3-
[(cholamidopropyl)dimethylammonio]-1-propanesulphonate
(CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of
25   acids and bases of pH 3.5 to 10 (2% w/v) and a trace of
Bromophenol Blue:
         (c)   emptying the cassette of liquid, transferring
the strips to an electrophoretic tray fitted with humid
electrode wicks, electrodes and sample cups, covering the
30   strips and cups with low viscosity paraffin oil;
         (d)   applying 200 micrograms of an aqueous solution
of dried, powdered material of the relevant body tissue
in urea (8M), CHAPS (4% w/v), Tris (40(mM), DTE (65mM),

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SDS (0.05% w/v) and a trace of Bromophenol Blue to the
sample cups, at the cathodic end of the IPG strips;

(e)   carrying out isoelectric focusing on the gel at
a voltage which increases linearly from 300 to 3500V
5   during 3 hours, followed by another 3 hours at 3500V, and
thereafter at 5000V for a time effective to enable the
proteins to migrate in the strips to their pH-dependent
final positions;

(f)   equilibrating the strips within the tray with
10   100ml of an aqueous solution containing Tris-HCl (50mM)
pH 6.8, urea (6M), glycerol (30% v/v), SDS (2% w/v) and
DTE (2% w/v) for 12 minutes;

(g)   replacing this solution by 100ml of an aqueous
solution containing Tris-HCl (50mM) pH 6.8, urea (6M),
15   glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5%
w/v) and a trace of Bromophenol Blue for 5 minutes;

(h)   providing a vertical gradient slab gel 160 x 200 x
1.5mm of acrylamide/piperazine-diacrylyl cross-linker (9-
16%T/2.6%C), polymerised in the presence of TEMED (0.5%
20   w/v), ammonium persulphate (0.1% w/v) and sodium
thiosulphate (5mM), in Tris-HCl (0.375M) pH 8.8 as
leading buffer;

(i)   over-layering the gel with sec-butanol for
about 2 hours, removing the overlay and replacing it with
25   water;

(j)   cutting the IPG gel strips to a size suitable
for the second dimensional electrophoresis, removing 6mm
from the anode end and 14mm from the cathode end;

(k)   over-laying the slab gel with an equeous
30   solution of agarose (0.5% w/v) and Tris-glycine-SDS
(25mM-198mM - 0.1% w/v) as leading buffer, heated to 70°C
and loading the IPG gel strips onto the slab gel through
this over-layered solution;

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(1)   running the second dimensional electrophoresis
at a constant current of 40mM at 8-12°C for 5 hours; and,

(m)   washing the gel.

5   Examples of differentially expressed proteins are
described in the examples below.

Alternatively, fingerprint proteins may be used in
methods for identifying compounds useful for the
10   treatment of body weight and/or eating disordered states.
'Target protein', as used herein, refers to a
differentially expressed protein involved in body weight
regulation and/or the control feeding such that
modulation of the expression of that protein may act to
15   prevent or ameliorate the body weight and/or eating
disordered state including, but not limited to, obesity.

This invention is based, in part, on systematic search
strategies involving modulations of body weight and/or
20   eating behaviours and obesity experimental paradigms,
coupled with sensitive detection of proteins by 2D-
electrophoresis.

The invention further provides methods for the
25   identification of compounds that modulate the expression
of proteins involved in body weight and feeding processes
relevant to the regulation of energy balance. Still
further, the present invention describes methods for the
prevention and/or treatment of obesity, which may involve
30   the administration of such compounds to individuals
predisposed to or exhibiting obesity.

Additionally, the present invention describes methods for

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the prognostic and diagnostic evaluation of subjects with
body weight and eating disordered states in order to make
a prognosis of the most effective therapy for each
subject.

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The examples presented below demonstrate the successful
use of the experimental paradigms of the invention to
identify target proteins associated with a body weight
and/or eating disordered state.

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Definitions

"Differential expression", as used herein, refers to at
least one recognisable difference in tissue protein
expression. It may be a quantitatively measurable, semi-
15   quantitatively estimatable or qualitatively detectable
difference in tissue protein expression. Thus, a
differentially expressed protein (herein DEP) may be
strongly expressed in tissue in the normal state and less
strongly expressed or not expressed at all in tissue in
20   the body weight or eating disordered state. Conversely,
it may be strongly expressed in tissue in the disorder
state but less strongly expressed or not expressed at all
in the normal state. Similarly the differential
expression can be either way around in the comparison
25   between untreated and treated tissue. Further,
expression may be regarded as differential if the protein
undergoes any recognisable change between the two states
under comparison.

30   Thus, in contrast to prior art methods as described in US
Patent No:5,702,902 which identify differentially
expressed genes by examining RNA, mRNA or cDNA libraries
derived from different tissue types, the present

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invention is based on methods which directly determine
differentially expressed proteins present in tissue
samples, by employing techniques such as 2D gel
electrophoresis.

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The term "paradigm" means a prototype example, test model
or standard.

Wherever a differentially expressible protein is used in
10   the screening procedure, it follows that there must have
been at some time in the past a preliminary step of
establishing a paradigm by which the differential
expressibility of the protein was pre-determined. Once
the paradigm has been established, it need not be re-
15   established on every occasion that a screening procedure
is carried out. The term "establishing a paradigm" is to
be construed accordingly.

Body weight and/or eating disordered state includes
20   conditions in which the body mass is either below normal
or above normal and conditions in which eating behaviour
is abnormal. Conditions characterised by body weight
and/or eating disordered state include obesity, anorexia,
bulimia and cathexic states associated with cancer, AIDS
25   and trauma.

Nutrient intake status includes any paradigm providing a
difference in nutrient intake such as differences between
fed and fasting, over-feeding v normal feeding, meal
30   eating v ad-libitum eating, sated v unsated.

Macronutrient selection includes paradigms in which
individuals or animals select or are provided different

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macronutrients such as high fat v low fat, fat preferring v carbohydratre preferring.

5   "Relevant tissue" means any tissue which undergoes a biological change in the body weight or eating disordered state or the experimental paradigm.

"Tissue ..: representative of ... subjects" means any tissue in which the above-mentioned biological change can be
10 simulated for laboratory purposes and includes, for example, a primary cell culture or cell line derived ultimately from relevant tissue.

The term "subjects" includes human and animal subjects.
15

The treatments referred to above can comprise the administration of one or more drugs or foodstuffs, and/or other factors such as diet or exercise.

20 The differentially expressed proteins (DEPs) include "fingerprint proteins", "target proteins" or "pathway proteins".

The term "fingerprint protein", as used herein, means a
25 DEP, the expression of which can be used, alone or together with other DEPs, to monitor or assess the condition of a patient suspected of suffering from a bodyweight or eating disordered state. Since these proteins will normally be used in combination, especially
30 a combination of four or more, they are conveniently termed "fingerprint proteins", without prejudice to the possibility that on occasions they may be used singly or along with only one or two other proteins for this

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purpose.   Such a fingerprint protein or proteins can be
used, for example, to diagnose a particular type of body
weight or eating disorder and thence to suggest a
specific treatment for it.

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The term "diagnosis", as used herein, includes the
provision of any information concerning the existence,
non-existence or probability of the disorder in a
patient.   It further includes the provision of
10   information concerning the type or classification of the
disorder or of symptoms, which are or may be experienced
in connection with it.   It encompasses prognosis of the
medical course of the disorder.

15   The term "target protein", as used herein, means a DEP,
the level or activity of which can be modulated by
treatment to alleviate a body weight or eating disordered
state.   Modulation of the level or activity of the target
protein in a patient may be achieved, for example, by
20   administering the target protein, another protein or gene
which interacts with it, or an agent which counteracts or
reduces it, for example an antibody to the protein,
competitive inhibitor of the protein or an agent which
acts in the process of transcription or translation of
25   the corresponding gene.

Alternatively or additionally, the DEPs can interact with
at least one other protein or which a gene involved in
the regulation of body weight or eating.   Such other
30   proteins are termed herein "pathway proteins" (PPs).   The
term is applied to the protein with which it, the DEP,
interacts, not the DEP itself, although a pathway protein
can be another DEP.

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By way of example, embodiments of the present invention
will now be described in more detail with reference to
the accompanying figures.

5   **Brief Description of the Figures**
Figures 1A-E show computer images of stained 2-DGE gels
from the liver tissue of lean mice identifying spots
thereon, including DEPs.

10   Figures 2-8 show DEPs which are underexpressed in ob/ob
mouse liver tissue relative to expression in lean mouse
lean mouse liver tissue.

Figures 9-11 show DEPs which are underexpressed in ob/ob
15   mouse skeletal muscle relative to expression in lean
mouse skeletal muscle, and DEPs which are overexpressed
in ob/ob skeletal muscle relative to lean mouse skeletal
muscle.

20   Figures 12-23 show DEPs which are underexpressed in ob/ob
mouse adipose tissue relative to lean mouse adipose
tissue, and DEPs which are overexpressed in obese mouse
adipose tissue relative to adipose tissue of lean mice.

25   Figures 24-29 show DEPs which are underexpressed in ob/ob
mouse brown adipose tissue relative to lean mouse brown
adipose tissue, and DEPs which are overexpressed in obese
mouse brown adipose tissue relative to brown adipose
tissue of lean mice.

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Detailed Description
Methods and compositions for the treatment of body weight
and/or eating disordered states including, but not

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limited to, obesity. Proteins termed 'target proteins' and/or fingerprint proteins are described which are differentially expressed in body weight and/or eating disordered states relative to their expression in normal

5   states and/or which are differentially expressed in response to manipulations relevant to the regulation of body weight and/or eating. Additionally, proteins termed 'pathway proteins' are described which interact with proteins involved in regulation of body weight and/or

10   eating. Methods for the identification of such fingerprint target and pathway proteins are also described.

Described below are methods for the identification of

15   compounds, which modulate the expression of proteins, involved in the regulation of body weight and/or eating. Additionally described below are methods for the treatment of body weight and/or eating disordered states including, but not limited to, obesity.

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Also discussed below are methods for prognostic and diagnostic evaluation of body weight and/or eating disordered states and for the identification of subjects exhibiting a predisposition to such disorders and for

25   identifying the most appropriate therapy for such individuals.

1.   **Identification of differentially expressed and pathway proteins**

30   In one embodiment, the present invention concerns methods for the identification of proteins which are involved in body weight and/or eating disorders and/or which are involved in obesity. Such proteins may represent

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proteins, which are differentially expressed in body
weight and/or eating disordered states relative to their
expression in normal states. Further, such proteins may
represent proteins that are differentially expressed or

5   regulated in response to manipulation relevant to
modulating body weight and/or food intake. Such
differentially expressed proteins may represent 'target'
or 'fingerprint' proteins. Methods for the
identification of such proteins are described in section

10   1.1. Methods for the further characterisation of such
differentially expressed proteins and for their
identification as target and/or fingerprint proteins are
presented below in section 1.3.

15   In addition, methods are described herein in section 1.3,
for the identification of proteins termed pathway
proteins involved in body weight and/or eating disordered
states and/or obesity. Pathway proteins, as used herein,
refer to a protein, which exhibits the ability to

20   interact with other proteins relevant to body weight
and/or eating disordered states. A pathway protein may
be differentially expressed and therefore may have the
characteristics of a target or fingerprint protein.

25   'Differential expression', as used herein, refers to both
qualitative as well as quantitative differences in
protein expression. Thus a differentially expressed
protein may qualitatively have its expression activated
or completely inactivated in normal versus body weight

30   and/or eating disordered state or under control versus
experimental conditions. Such a qualitatively regulated
protein will exhibit an expression pattern within a given
tissue or cell type, which is detectable in either

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control or body weight and/or eating disordered subject,
but not detectable in both. Alternatively, such a
qualitatively regulated protein will exhibit an
expression pattern within one or more cell types, which
5   is detectable in either control or experimental subjects
but not detectable in both. 'Detectable', as used
herein, refers to a protein expression pattern, which is
detectable by the technique of differential display 2D
electrophoresis, which are well known to those of skill
10   in the art.

Alternatively, a differentially expressed protein may
have its expression modulated, i.e. quantitatively
increased or decreased, in normal versus body weight or
15   eating disorder states or under control versus
experimental conditions. The degree to which expression
differs in normal versus body weight and/or eating
disordered states or control versus experimental states
need only be large enough to be visualised via standard
20   characterisation techniques, such as silver staining of
2D-electrophoretic gels. Other such standard
characterisation techniques by which expression
differences may be visualised are well known to those
skilled in the art. These include successive
25   chromatographic separations of fractions and comparisons
of the peaks, capillary electrophoresis and separations
using micro-channel networks, including on a microchip.

Chromatographic separations can be carried out by high
30   performance liquid chromatography as described in
Pharmacia literature, the chromatogram being obtained in
the form of a plot of absorbance of light at 280nm
against time of separation. The material giving

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incompletely resolved peaks is then re-chromatographed
and so on.

Capillary electrophoresis is a technique described in
many publications, for example in the literature "Total
CE Solutions" supplied by Beckman with their P/ACE 5000
system. The technique depends on applying an electric
potential across the sample contained in a small
capillary tube. The tube has a charged surface, such as
negatively charged silicate glass. Oppositely charged
ions (in this instance, positive ions) are attracted to
the surface and then migrate to the appropriate electrode
of the same polarity as the surface (in this instance,
the cathode). In this electro-osmotic flow (EOF) of the
sample, the positive ions move fastest, followed by
uncharged material and negatively charged ions. Thus,
proteins are separated essentially according to charge on
them.

Micro-channel networks function somewhat like capillaries
and can be formed by photoablation of a polymeric
material. In this technique, a UV laser is used to
generate high energy light pulses that are fired in
bursts onto polymers having suitable UV absorption
characteristics, for example polyethylene terephthalate
or polycarbonate. The incident photons break chemical
bonds with a confined space, leading to a rise in
internal pressure, mini-explosions and ejection of the
ablated material, leaving behind voids which form micro-
channels. The micro-channel material achieves a
separation based on EOF, as for capillary
electrophoresis. It is adaptable to micro-chip form,
each chip having its own sample injector, separation

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column and electrochemical detector: see J.S. Rossier *et al*, 1999, Electrophoresis *20*: pp. 727-731.

Differentially expressed proteins may be further
described as target proteins and/or fingerprint proteins.
'Fingerprint proteins', as used herein, refer to a
differentially expressed protein whose expression pattern
may be utilised as part of a prognostic or diagnostic
body weight and/or eating disorder evaluation or which,
alternatively, may be used in methods for identifying
compounds useful for the treatment of body weight and/or
eating disordered states. A fingerprint protein may also
have characteristics of a target protein or a pathway
protein.

'Target protein', as used herein, refers to a
differentially expressed protein involved in body weight
and/or eating disordered states and/or obesity such that
modulation of the level or activity of the protein may
act to prevent the development of the disordered states
including, but not limited to, obesity. A target protein
may also have the characteristics of a fingerprint
protein or a pathway protein.

25   **1.1 Method for the identification of differentially**
expressed proteins

A variety of methods may be used for the identification
of proteins, which are involved in body weight and/or
eating disordered states and/or which may be involved in
obesity. Described in Section 1.1.1 are several
experimental paradigms, which may be utilised for the
generation of subjects, and samples, which may be used
for the identification of such proteins. Material from

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the paradigm control and experimental subjects may be
characterised for the presence of differentially
expressed protein sequences as discussed below in Section
1.1.2.

5

1.1.1   Paradigms for the identification of
differentially expressed proteins
Among the paradigms that may be utilised for the
identification of differentially expressed proteins
10   involved in body weight and/or obesity disordered states
are paradigms designed to analyse those proteins that are
differentially expressed between normal and body weight
and/or eating disordered states including, but not
limited to, obesity.

15

In one embodiment of such a paradigm tissue from normal
and body weight and/or eating disorder subjects would be
compared. Such subjects could include, but would not be
limited to, subjects with obesity. It could also involve
20   a comparison of normal subjects and subjects who have
resisted the development of obesity despite overfeeding.
Appropriate tissues would include, but not be limited to,
blood and adipose tissue. It could also include post-
mortem samples from subjects. Particularly useful
25   tissues would include brain particularly the
hypothalamus, skeletal muscle, liver and adipose tissue.

Among additional paradigms would include a comparison of
obese subjects and obese subjects whose body weight had
30   been reduced by, but not limited to, dietary restriction
or dietary modification, anorexic drugs such as
sibutramine, Y5 receptor antagonists, leptin and leptin
mimetics, MC4 agonists, exercise and thermogenic drugs

such as b₃-adrenoceptor agonists.

Among further paradigms would include a comparison of
adipose tissue from various fat depots within the same
individuals but typically to be a comparison of
subcutaneous and omental adipose tissue since omental
adipose tissue is associated with the metabolic and
cardiovascular complications of obesity, whereas excess
subcutaneous adipose tissue does not carry a similar high
risk.

In a further paradigm, which may be utilised for the
identification of differentially expressed proteins
involved in body weight and/or eating disorders are
paradigms designed to analyse those proteins which may be
involved in genetic models of obesity. Accordingly, such
paradigms are referred to as 'genetic obesity paradigms'.
In the case of mice, for example, such paradigms may
identify the proteins regulated either directly or
indirectly by the ob/ob, db/db, tub or fat gene products.
In rats such a paradigm may identify proteins regulated
either directly or indirectly by the fa gene product.

In one embodiment of such a paradigm, test subjects may
include ob/ob, db/db, tub/tub or fat/fat experimental
mice and lean littermate controls. Test subjects could
also include fa/fa and male and female ZDF rats. Samples
of tissues such as skeletal muscle, whole brain,
hypothalamus, adipose tissue and liver would be obtained.
The examples below demonstrate the use of such genetic
paradigms in identifying proteins which are
differentially expressed in obese animals versus normal
animals.

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In additional embodiments, ob/ob, db/db, tub/tub and/or
fat/fat mice and/or fa/fa and ZDF rats and lean control
animals and inbred or outbred strains of rodents may be
treated with drugs that reduce body weight, particularly

5   body fat mass. Such drugs include, but are not limited
to, appetite suppressants such as sibutramine,
fenfluramine, NPY antagonists, melanocortin-4 receptor
agonists, orexin antagonists, MCH antagonists,
thermogenic drugs such as b_3-adrenoceptor agonists and

10   anti-obesity agents such as leptin, leptin mimetics and
other cytokines such as axokine and brain derived
neurotrophic factor. Such a paradigm allows the
identification of target proteins.

15   In a further additional embodiment, ob/ob, db/db, tub/tub
and/or fat/fat mice and/or fa/fa and/or ZDF rats and lean
controls or inbred or outbred strains of rodents may be
offered dietary treatments to either worsen the obese
state or reduce the level of obesity or to identify

20   animals that resist dietary obesity. For example, either
lean or obese animals could be provided with a high fat
diet to exacerbate the obese state.

In one embodiment of such a paradigm, Sprague Dawley rats

25   would be fed on a high fat diet or a cafeteria diet
consisting of human snack foods. Some of the rats become
obese whilst others resist obesity. Thus, this paradigm
can be used to select proteins that are associated with a
predisposition to the development of obesity and to

30   proteins associated with an ability to resist dietary
obesity. This paradigm can be further refined by
incorporating drug treatment or exercise paradigms.

In another embodiment of this paradigm, obese rodents
that had been weight reduced by dietary restriction would
be allowed access to ad-libitum food whilst a control
group of obese rodents would remain dietary restricted.

5   Such a paradigm would allow the identification of
proteins associated with the regain of obesity following
withdrawal of dietary restriction.

Some native animal strains do not exhibit obesity in the
10   wild but do when fed a laboratory chow or other
laboratory diets. These include the desert rodents, the
spiny mouse and the sand rat. Comparison of animals fed
on the natural diet and those fed on a laboratory diet
allows identification of proteins associated with body
15   weight disorders and obesity.

Selective breeding can also be used to obtain genetically
closely related animals that exhibit different degrees of
adiposity and/or metabolic efficiency. Comparisons
20   between these closely related animals allows the
identification of proteins associated with body weight
disorders and obesity.

A particularly important tissue with respect to the
25   control of feeding behaviour and the regulation of energy
balance is the hypothalamus. It is clear that many
substances act in this area of the brain to regulate
feeding and/or energy balance. These include
catecholamines, serotinergic agents, leptin, orexins,
30   melanocyte concentrating hormone, melanocortin receptor
agonists and antagonists. It is also clear that there
are complex interactions between these various
substances.

with differentially expressed proteins which are relevant
to obesity. A pathway protein may be differentially
expressed and, therefore, may have the characteristics of
a target and/or fingerprint protein.

5

Any method suitable for detecting protein-protein
interactions may be employed for identifying pathway
proteins by identifying interactions between proteins and
proteins known to be differentially expressed in body

10   weight and/or eating disordered states and/or obesity
regulation. Such differentially expressed proteins may
be cellular or extracellular proteins. Those proteins,
which interact with such differentially expressed
proteins, represent pathway gene products.

15

Among the traditional methods, which may be employed, are
co-immunoprecipitation, crosslinking and co-purification
through gradients or chromatographic columns. Utilising
procedures such as these allows for the identification of

20   pathway proteins. Once identified, a pathway protein may
be used, in conjunction with standard techniques, to
identify its corresponding pathway gene. For example, at
least a portion of the amino acid sequence of the pathway
gene product may be ascertained using techniques well

25   known to those of skill in the art, such as via the Edman
degradation technique (see, e.g. Creighton (1983)
'Proteins: Structures and Molecular Principles', W.H.
Freeman & Co., N.Y., pp. 34-49). The amino acid sequence
obtained may be used as a guide for the generation of

30   oligonucleotide mixtures that can be used to screen for
pathway gene sequences. Screening may be accomplished,
for example, by standard hybridisation or PCR techniques.
Techniques for the generation of oligonucleotide mixtures

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However, no systematic evaluation of the changes in protein composition of the hypothalamus and its constituent nuclei, which underlay the changes in feeding behaviour, has yet been undertaken. Thus, in a further embodiment, hypothalamic nuclei from fed v fasted rodents; sated v non-sated rodents; obese v lean rodents; fat preferring v non-fat preferring rodents may be used to identify the hypothalamic protein patterns associated with particular feeding behaviours.

In addition to whole animal studies, paradigms include systems in which isolated cells such as adipocytes, are incubated in vitro with agents such as leptin.

1.1.2   Analysis of paradigm material
In order to identify differentially expressed proteins, tissues from subjects utilised in paradigms such as those described above in 1.1.1 are obtained. In addition, blood and body fluids may be analysed since the differentially expressed proteins might be released into the circulations.

Whole tissue or isolated cells may be used. Sub-cellular fractions of cells might also be used. Particularly useful sub-cellular fractions include the nuclear protein fraction.

1.2   Methods for the identification of pathway proteins
Methods are described herein for the identification of pathway proteins. 'Pathway protein', as used herein, refers to a protein which exhibits the ability to interact with differentially expressed proteins involved in body weight and/or eating disorders and/or to interact

and the screening are well-known (see, e.g. Ausubel,
supra. and PCR Protocols: A Guide to Methods and
Applications (1990) Innis, M. et al., eds. Academic Press
Inc., New York).

5

One method, which detects protein interactions in vivo,
the yeast two-hybrid system, is described in detail for
illustration only and not by way of limitation. One
version of this system has been described (Chien et al
10   (1991) Proc. Natl. Acad. Sci. USA, 88, 9578-9582) and is
commercially available from Clontech (Palo Alto, Calif.).

Briefly, utilising such a system, plasmids are
constructed that encode two hybrid proteins: one
15   consists of the DNA-binding domain of a transcription
activator protein fused to a known protein, in this case,
the differentially expressed protein known to be involved
in body weight and/or eating disordered states and/or
obesity regulation, and the other consists of the
20   transcription activator protein's activation domain fused
to an unknown protein that is encoded by a cDNA which has
been recombined into this plasmid as part of a cDNA
library. The plasmids are transformed into a strain of
the yeast Saccharomyces cerevisiae that contains a
25   reporter gene (e.g. lacZ) whose regulatory region
contains the transcription activator's binding sites.
Either hybrid protein alone cannot activate transcription
of the reporter gene: the DNA-binding domain hybrid
cannot because it does not provide activation function
30   and the activation domain hybrid cannot because it cannot
localise to the activator's binding sites. Interaction
of the two hybrid proteins reconstitutes the functional
activator protein and results in expression of the

reporter gene, which is detected by an assay for the
reporter gene product.

The yeast two-hybrid system or related methodology may be
used to screen activation domain libraries for proteins
that interact with a known differentially expressed
'bait' protein. Total genomic or cDNA sequences are
fused to the DNA encoding an activation domain. This
library and a plasmid encoding a hybrid of the bait
protein product fused to the DNA-binding domain are co-
transformed into a yeast reporter strain, and the
resulting transformants are screened for those that
express the reporter gene. For example, and not by way
of limitation, the bait gene can be cloned into a vector
such that it is translationally fused to the DNA encoding
the DNA-binding domain of the GAL4 protein. These
colonies are purified and the library plasmids
responsible for reporter gene expression are isolated.
DNA sequencing is then used to identify the proteins
encoded by the library plasmids.

A cDNA library of the cell line from which proteins that
interact with bait proteins are to be detected can be
made using methods routinely practised in the art.
According to the particular system described herein, for
example, the cDNA fragments can be inserted into a vector
such that they are translationally fused to the
activation domain of GAL4. This library can be co-
transformed along with the bait-gene GAL4 fusion plasmid
into a yeast strain, which contains a lacZ gene driven by
a promoter which contains GAL4 activation sequence. A
cDNA encoded protein, fused to GAL4 activation domain,
that interacts with bait gene product will reconstitute

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an active GAL4 protein and thereby drive expression of
the lacZ gene. Colonies which express lacZ can be
detected by their blue color in the presence of X-gal.
The cDNA can then be purified from these strains, and
5   used to produce and isolate the bait gene-interacting
protein using techniques routinely practised in the art.

Once a pathway protein has been identified and isolated,
it may be further characterised as, for example,
10   discussed below, in Section 1.3.

1.3   **Characterisation of differentially expressed and
pathway proteins**
Differentially expressed proteins, such as those
15   identified via the methods discussed, above, in Section
1.1, and pathway genes, such as those identified via the
methods discussed, above, in Section 1.2, above, as well
as genes identified by alternative means, may be further
characterised by utilising, for example, methods such as
20   those discussed herein. Such proteins will be referred
to herein as 'identified proteins'.

Analyses such as those described herein, yield
information regarding the biological function of the
25   identified proteins. An assessment of the biological
function of the differentially expressed proteins, in
addition, will allow for their designation as target
and/or fingerprint proteins.

30   Specifically, any of the differentially expressed
proteins whose further characterisation indicates that a
modulation of the proteins expressed or a modulation of
the proteins activity may ameliorate any of the body

weight and/or eating disorders will be designated 'target

proteins', as defined above, in Section 1. Such target

proteins, along with those discussed below, will

constitute the focus of the compound discovery strategies

5   discussed below, in Section 3. Further, such target

proteins and/or modulating compounds can be used as part

of the treatment and/or prevention of body weight and/or

eating disorders and/or obesity.

10   Any of the differentially expressed proteins whose

further characterisation indicates that such modulations

may not positively affect body weight and/or eating

disorders, but whose expression pattern contributes to a

protein 'fingerprint' pattern correlative of, for

15   example, a body weight and/or eating disordered state,

will be designated a 'fingerprint protein'. 'Fingerprint

patterns' will be more fully discussed below, in Section

7.1. It should be noted that each of the target proteins

may also function as fingerprint proteins, as well as may

20   all or a portion of the pathway proteins.

It should further be noted that the pathway proteins may

also be characterised according to techniques such as

those described herein. Those pathway proteins which

25   yield information indicating that they are differentially

expressed and that modulation of the proteins expression

or a modulation of the proteins expression or a

modulation of the proteins activity may ameliorate any of

the body weight and/or eating disorders of interest will

30   also be designated 'target proteins'. Such target

proteins, along with those discussed above, will

constitute the focus of the compound discovery strategies

discussed below, in Section 3 and can be used as part of

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the treatment methods described in Section 4, below.

It should be additionally noted that the characterisation
of one or more of the pathway proteins may reveal a lack
5   of differential expression, but evidence that modulation
of the gene's activity or expression may, nonetheless,
ameliorate body weight and/or eating disorder symptoms.
In such cases, these genes and gene products would also
be considered a focus of the compound discovery
10   strategies of Section 3 below.

In instances wherein a pathway proteins characterisation
indicates that modulation of gene expression or gene
product activity may not positively affect body weight
15   and/or eating disorders of interest, but whose expression
is differentially expressed and contributes to a gene
expression fingerprint pattern correlative of, for
example, a body weight and/or eating disordered state,
such pathway genes may additionally be designated as
20   fingerprint genes.

A variety of techniques can be utilised to further
characterise the identified proteins. First, the
corresponding nucleotide sequence of the identified
25   protein may be obtained by utilising standard techniques
well known to those of skill in the art, may, for
example, be used to reveal homologies to one or more
known sequence motifs which may yield information
regarding the biological function of the identified
30   protein.

Secondly, the biological function of the identified
proteins may be more directly assessed by utilising

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relevant in vivo and in vitro systems. In vivo systems
may include, but are not limited to, animal systems,
which naturally exhibit body weight and/or eating
disorder-like symptoms, or ones which have been
engineered to exhibit such symptoms. Further, such
systems may include systems for the further
characterisation of body weight and/or eating disorders,
and/or obesity, and may include, but are not limited to,
naturally occurring and transgenic animal systems such as
those described above, in Section 1.1.1, and Section
2.2.1 below. In vitro systems may include, but are not
limited to, cell-based systems comprising cell types
known to be associated with energy storage and
metabolism. Such cells may be wild type cells, or may be
non-wild type cells containing modifications known to, or
suspected of, contributing to a body weight a /or eating
disorder of interest. Such systems are discussed in
detail below, in Section 2.2.2.

In further characterising the biological function of the
identified proteins, the expression of these proteins may
be modulated within the in vivo and/or in vitro systems,
i.e. either overexpressed or underexpressed in, for
example, transgenic animals and/or cell lines, and its
subsequent effect on the system then assayed.
Alternatively, the activity of the identified protein may
be modulated by either increasing or decreasing the level
of activity in the in vivo and/or in vitro system of
interest, and its subsequent effect then assayed.

The information obtained through such characterisations
may suggest relevant methods for the treatment of body
weight and/or eating disorders involving the protein of

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interest. Further, relevant methods for the control of
obesity involving the protein of interest may be
suggested by information obtained from such
characterisations. For example, treatment may include a
5   modulation of protein expression and/or protein activity.
Characterisation procedures such as those described
herein may indicate where such modulation should involve
an increase or a decrease in the expression or activity
of the protein of interest. Such methods of treatment
10   are discussed below, in Section 4.

2.   Differentially expressed and pathway proteins
Identified proteins, which include, but are not limited
to, differentially expressed proteins such as those
15   identified in Section 1.1 above, and pathway proteins,
such as those identified in Section 1.2 above, are
described herein. Specifically, the amino acid sequences
of such identified proteins are described. Further,
antibodies directed against the identified protein, and
20   cell- and animal-based models by which the identified
proteins may be further characterised and utilised are
also discussed in this Section.

2.1 Antibodies specific for differentially expressed or
25   pathway proteins
Described herein are methods for the production of
antibodies capable of specifically recognising one or
more differentially expressed or pathway protein
epitopes. Such antibodies may include, but are not
30   limited to, polyclonal antibodies, monoclonal antibodies
(mAbs), humanised or chimeric antibodies, single chain
antibodies, Fab fragments, F(ab')$_2$ fragments, fragments
produced by a FAb expression library, anti-idiotypic

(anti-Id) antibodies, and epitope-binding fragments of
any of the above. Such antibodies may be utilised as
part of body weight and/or eating disorder treatment
methods, and/or may be used as part of diagnostic
5   techniques whereby patients may be tested for abnormal
levels of fingerprint, target, or pathway gene proteins,
or for the presence of abnormal forms of such proteins.

For the production of antibodies to a differentially
10   expressed or pathway protein, various host animals may be
immunised by injection with a differentially expressed or
pathway protein, or a portion thereof. Such host animals
may include, but are not limited to, rabbits, mice and
rats, to name but a few. Various adjuvants may be used
15   to increase the immunological response, depending on the
host species, including active substances such as
lysolecithin, pluronic polyols, polyanions, peptides, oil
emulsions, keyhole limpet hemocyamin, dinitrophenol, and
potentially useful human adjuvant such as BCG bacille
20   Calmette-Fuerin) and Corynebacterium parvum.

Polyclonal antibodies are heterogeneous populations of
antibody molecules derived from the sera of animals
immunised with an antigen, such as target proteins, or an
25   antigenic functional derivative thereof. For the
production of polyclonal antibodies, host animals such as
those described above, may be immunised by injection with
differentially expressed or pathway protein supplemented
with adjuvants as also described above.
30
Monoclonal antibodies, which are homogeneous populations
of antibodies to a particular antigen, may be obtained by
any technique, which provides for the production of

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antibody molecules by continuous cell lines in culture.
These include, but are not limited to, the hybridoma
technique of Kohler and Milstein (1975, Nature 256; 495-
497; and US Pat. No. 4,376,110), the human b-cell

5   hybridoma technique (Kosbor, et al., 1983, Immunology
Today 4: 72; Cole, et al., 1983, Proc. Natl. Acad. Sci.
USA 80; 2026-2030), and the EBV-hybridoma technique
(Cole, et al., 1985, Monoclonal Antibodies and Cancer
Therapy, Alan R. Liss Inc., pp. 77-96). Such antibodies

10   may be of any immunoglobulin class including IgG, IgM,
IgE, IgA, IgD and any subclass thereof. The hybridoma
producing the mAb of this invention may be cultivated in
vitro or in vivo. Production of high titers of mAbs in
vivo makes this the presently preferred method of

15   production.

In addition, techniques developed for the production of
'chimeric antibodies' (Morrison, et al., 1984, Proc.
Natl. Acad. Sci. 81: 6851-6855; Neuberger, et al., 1984,

20   Nature 312: 604-608; Takeda, et al., 1985, Nature 314:
452-454) by splicing the genes from a mouse antibody
molecule of appropriate antigen specificity together with
genes from a human antibody molecule of appropriate
biological activity can be used. A chimeric antibody is

25   a molecule in which different portions are derived from
different animal species, such as those having a variable
region derived from a murine mAb and a human
immunoglobulin constant region.

30   Alternatively, techniques described for the production of
single chain antibodies (US Pat. No. 4,946,778; Bird,
1988, Science 242: 423-426; Huston, et al., 1988, Proc.
Natl. Acad. Sci. USA 85: 5879-5883; and Ward, et al.,

1989, Nature 334: 544-546) can be adapted to produce
differentially expressed or pathway protein-single chain
antibodies. Single chain antibodies are formed by
linking the heavy and light chain fragments of the Fv

5   region via an amino acid bridge, resulting in a single
chain polypeptide.

Antibody fragments, which recognise specific epitopes,
may be generated by known techniques. For example, such

10   fragments include, but are not limited to, the F(ab')$_2$
fragments which can be produced by pepsin digestion of
the antibody molecule and the Fab fragments which can be
generated by reducing the disulfide bridges of the F(ab')$_2$
fragments. Alternative, Fab expression libraries may be

15   constructed (Huse, et al., 1989, Science 246: 1275-1281)
to allow rapid and easy identification of monoclonal Fab
fragments with the desired specificity.

2.2   Cell- and animal-based model systems

20   Described herein are cell- and animal-based systems,
which act as models for body weight and or eating
disorders. These systems may be used in a variety of
applications. For example, the animal-based model
systems can be utilised to identify differentially

25   expressed proteins via one of the paradigms described
above, in Section 1.1.1. Cell- and animal-based model
systems may be used to further characterise
differentially expressed and pathway proteins, as
described above in Section 1.3. Such further

30   characterisation may, for example, indicate that a
differentially expressed protein is a target protein.
Second, such assays may be utilised as part of screening
strategies designed to identify compounds which are

capable of ameliorating body weight and/or eating
disorder symptoms, as described below. Thus, the animal-
and cell-based models may be used to identify drugs,
pharmaceuticals, therapies and interventions which may be
5   effective in treating such body weight and/or eating
disorders. In addition, as described in detail below, in
Section 6, such animal models may be used to determine
the LD_{50} and the ED_{50} in animal subjects, and such data can
be used to determine the in vivo efficacy of potential
10   body weight and/or eating disorder treatments, including
treatments for obesity.

2.2.1   Animal-based systems

Animal-based model systems of body weight and/or eating
15   disorders may include, but are not limited to, non-
recombinant and engineered transgenic animals.

Non-recombinant animal models for body weight and/or
eating disorders may include, for example, genetic
20   models. Such genetic body weight and/or eating disorder
models may include, for example, mouse models of obesity
such as mice homozygous for the autosomal recessive ob,
db, fat or tub alleles. It could also include rat
models, for example fa/fa rats.

25

Non-recombinant, non-genetic animal models of body weight
and/or eating disorder may include, for example, rats or
mice fed on a diet containing a large amount of fat.
Such diets could be synthetic diets in which the fat
30   content (by calorific value) is more than 50%.
Alternative human foods with a high fat content, such as
salami and butter, may be provided to the animals.

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Additionally, animal models exhibiting body weight and/or eating disorder-like symptoms may be engineered by utilising, for example, the gene sequences of target proteins such as those described above, in Section 2, in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art. For example, gene sequences of target proteins may be introduced into, and overexpressed in, the genome of the animal of interest, or, if endogenous gene sequences of target proteins are present, they may either be overexpressed or, alternatively, may be disrupted in order to underexpress or inactivate gene expression of target proteins.

In order to overexpress the target gene sequence of a target protein, the coding portion of the target gene sequence may be ligated to a regulatory sequence, which is capable of driving gene expression in the animal and cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilised in the absence of undue experimentation.

For underexpression of an endogenous gene sequence of a target protein, such a sequence may be isolated and engineered such that when reintroduced into the genome of the animal of interest, the endogenous gene alleles of the target protein will be inactivated. Preferably, the engineered gene sequence of the target protein is introduced via gene targeting such that the endogenous sequence is disrupted upon integration of the engineered target gene sequence into the animal's genome.

Animals of any species, including, but not limited to,

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mice, rats, rabbits, guinea pigs, pigs, mini-pigs, goats
and non-human primates, e.g. baboons, squirrel, monkeys,
rhesus monkeys and chimpanzees may be used to generate
body weight and/or eating disorder animal models.

Any technique known in the art may be used to introduce a
target gene transgene of a target protein into animals to
produce the founder lines of transgenic animals. Such
techniques include, but are not limited to, pronuclear
microinjection (Hoppe, P.C. and Wagner, T.E., 1989, US
Pat.No. 4,873,191); retrovirus mediated gene transfer
into germ lines (Van der Putten et al., 1985, Proc. Natl.
Acad. Sci., USA 82: 6148-6152); gene targeting in
embryonic stem cells (Thompson et al., 1989, Cell 56:
313-321); electroporation of embryos (Lo, 1983, Mol. Cell
Biol. 3: 1803-1814); and sperm-mediated gene transfer
(Lavitrano et al., 1989, Cell 57: 717-723); etc. For a
review of such techniques, see Gordon, 1989, Transgenic
Animals, Intl. Rev. Cytol. 115: 171-229.

The present invention provides for transgenic animals
that carry the transgene in all their cells, as well as
animals which carry the transgene in some, but not all
their cells, i.e. mosaic animals (see, for example,
techniques described by Jakobovits, 1994, Curr. Biol. 4:
761-763). The transgene may be integrated as a single
transgene or in concatamers, e.g. head-to-head tandems or
head-to-tail tandems. The transgene may also be
selectively introduced into and activated in a particular
cell type by following, for example, the teaching of
Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad.
Sci. USA 89: 6232-6236). The regulatory sequences
required to such a cell-type specific activation will

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depend upon the particular cell type of interest, and
will be apparent to those of skill in the art.

When it is desired that the target gene transgene be

5   integrated into the chromosomal site of the endogenous
target gene, gene targeting is preferred. Briefly, when
such a technique is to be utilised, vectors containing
some nucleotide sequences homologous to the gene of the
endogenous target protein of interest are designed for

10   the purpose of integrating, via homologous recombination
with chromosomal sequences, into and disrupting the
function of, the nucleotide sequence of the endogenous
target gene. The transgene may also be selectively
introduced into a particular cell type, thus inactivating

15   the endogenous gene of interest in only that cell type,
by following, for example, the teaching of Gu et al. (Gu,
H. et al., 1994, Science 265: 103-106). The regulatory
sequences required for such a cell-type specific
inactivation will depend upon the particular cell type of

20   interest, and will be apparent to those of skill in the
art.

Once transgenic animals have been generated, the
expression of the recombinant target gene and protein may

25   be assayed utilising standard techniques. Initial
screening may be accomplished by Southern blot analysis
or PCR techniques to analyse animal tissues to assay
whether integration of the transgene has taken place.
The level of mRNA expression of the transgene in the

30   tissues of the transgenic animals may also be assessed
using techniques which include, but are not limited to,
Northern blot analysis of tissue samples obtained from
the animal, in situ hybridisation analysis, and RT-PCR.

Samples of target protein-expressing tissue may also be
evaluated immunocytochemically using antibodies specific
for the transgene protein of interest.

5   The target protein transgenic animals that express target
gene mRNA or target protein transgene peptide (detected
immunocytochemically, using antibodies directed against
target protein epitopes) at easily detectable levels
should then be further evaluated to identify those
10   animals which display characteristic body weight and/or
eating disorder-like symptoms. Such symptoms may
include, for example, obesity, hyperphagia, hypophagia,
leanness, glucose intolerance, hyperinsulinaemia, and/or
non-insulin dependent diabetes. Additionally, specific
15   cell types within the transgenic animals may be analysed
and assayed for cellular phenotypes characteristic of
body weight and/or eating disorders. Further, such
cellular phenotypes may include an assessment of a
particular cell types fingerprint pattern of expression
20   and its comparison to known fingerprint expression
profiles of the particular cell type in animals
exhibiting body weight and/or eating disorders. Such
transgenic animals serve as suitable model systems for
body weight and/or eating disorders.

25

Once target protein transgenic founder animals are
produced (i.e. those animals which express target
proteins in cells or tissues of interest and which,
preferably, exhibit symptoms of body weight and/or eating
30   disorders), they may be bred, inbred, outbred or
crossbred to produce colonies of the particular animal.
Examples of such breeding strategies include, but are not
limited to, outbreeding of founder animals with more than

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one integration site in order to establish separate
lines; inbreeding of separate lines in order to produce
compound target protein transgenics that transgenically
express the target protein of interest at higher levels

5   because of the effects of additive expression of each
target gene transgene; crossing of heterozygous
transgenic animals to produce animals homozygous for a
given integration site in order to both augment
expression and eliminate the possible need for screening

10   of animals by DNA analysis; crossing of separate
homozygous lines to produce compound heterozygous or
homozygous lines; breeding animals to different inbred
genetic backgrounds so as to examine effects of modifying
alleles on expression of the target protein and the

15   development of body weight and/or eating disorder-like
symptoms. One such approach is to cross the target
protein transgenic founder animals with a wild type
strain to produce an F1 generation that exhibits body
weight and/or eating disorder-like symptoms, such as

20   hyperphagia, hypophagia, obesity and leanness. The F1
generation may then be inbred in order to develop a
homozygous line, if it is found that homozygous target
protein transgenic animals are viable.

25   **2.2.2   Cell-based assays**
Cells that contain and express target gene sequences
which encode target proteins and, further, exhibit
cellular phenotypes associated with an obesity disorder,
may be utilised to identify compounds that exhibit an

30   ability to ameliorate body weight and/or eating disorder
symptoms. Cellular phenotypes, which may indicate an
ability to ameliorate body weight and/or eating
disorders, may include, for example, resistance to

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insulin.

Further, cell lines which may be used for such assays may
also include recombinant, transgenic cell lines. For
example, the body weight and/or eating disorder animal
models of the invention discussed above, in Section
2.2.1, may be used to generate cell lines, containing one
or more cell types involved in body weight and/or eating
disorders, that can be used as cell culture models for
this disorder. While primary cultures derived from the
body weight and/or eating disorder transgenic animals of
the invention may be utilised, the generation of
continuous cell lines is preferred. For examples of
techniques which may be used to derive a continuous cell
line from the transgenic animals, see Small, et al.,
1985, Mol. Cell Biol. 5: 642-648.

Alternatively, cells of a cell type known to be involved
in body weight and/or eating disorders may be transfected
with sequences capable of increasing or decreasing the
amount of target protein within the cell. For example,
gene sequences of target proteins may be introduced into,
and overexpressed in, the genome of the cell of interest,
or, if endogenous gene sequences of the target protein
are present, they may either be overexpressed or,
alternatively, be disrupted in order to underexpress or
inactivate target protein expression.

In order to overexpress a gene sequence of a target
protein, the coding portion of the target gene sequence
may be ligated to a regulatory sequence, which is capable
of driving gene expression in the cell type of interest.
Such regulatory regions will be well known to those of

skill in the art, and may be utilised in the absence of
undue experimentation.

For underexpression of an endogenous target protein the
gene sequence may be isolated and engineered such that
when reintroduced into the genome of the cell type of
interest, the endogenous target gene alleles will be
inactivated. Preferably, the engineered target gene
sequence is introduced via gene targeting such that the
endogenous target sequence is disrupted upon integration
of the engineered target gene sequence into the cell's
genome. Gene targeting is discussed above, in Section
2.2.1.

Transfection of target protein gene sequence nucleic acid
may be accomplished by utilising standard techniques.
See, for example, Ausubel, 1989, supra. Transfected
cells should be evaluated for the presence of the
recombinant target gene sequences, for expression and
accumulation of target gene mRNA, and for the presence of
recombinant target protein production. In instances
wherein a decrease in target protein expression is
desired, standard techniques may be used to demonstrate
whether a decrease in endogenous target gene expression
and/or in target protein production is achieved.

**3.   Screening assays for compounds that interact with
the target proteins**

The following assays are designed to identify compounds
that bind to target proteins, bind to other cellular
proteins that interact with a target proteins, and to
compounds that interfere with the interaction of the
target proteins with other cellular proteins. Such

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compounds may include, but are not limited to, other
cellular proteins. Methods for the identification of
such cellular proteins are described below, in Section
3.2

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Compounds may include, but are not limited to, peptides
such as, for example, soluble peptides, including, but
not limited to, Ig-tailed fusion peptides, comprising
extracellular portions of target protein transmembrane
10   receptors, and members of random peptide libraries (see,
e.g. Lam, K.S. et al., 1991, Nature 354: 82-84;
Houghten, R. et al., 1991, Nature 354: 84-86) made of D-
and/or L-configuration amino acids, phosphopeptides
(including, but not limited to, member of random or
15   partially degenerate, directed phosphopeptide libraries:
se, e.g., Songyang, Z. et al., 1993, Cell 72: 767-778),
antibodies (including, but not limited to, polyclonal,
monoclonal, humanised, anti-idiotypic, chimeric or single
chain antibodies, and FAb, F(ab')$_2$ and FAb expression
20   library fragments, and epitope-binding fragments thereof)
and small organic or inorganic molecules.

Compounds identified via assays such as those described
herein may be useful, for example, in elaborating the
25   biological function of the target protein, and for
ameliorating body weight and/or eating disorders. In
instances, for example, whereby a body weight and/or
eating disorder situation results from a lower overall
level of target protein expression and/or target protein
30   activity in a cell or tissue involved in such a body
weight and/or eating disorder, compounds that interact
with the target protein may include ones which accentuate
or amplify the activity of the bound target protein. Such

compounds would bring about an effective increase in the
level of target protein activity, thus ameliorating
symptoms. In instances whereby mutations within the
target gene cause aberrant target proteins to be made

5   which have a deleterious effect that leads to a body
weight and/or eating disorder, compounds that bind target
protein may be identified that inhibit the activity of
the bound target protein. Assays for testing the
effectiveness of compounds, identified by, for example,

10   techniques such as those described in Section 3.1 to 3.3,
are discussed below, in Section 3.4.

3.1   **In vitro screening assays for compounds that bind to
the target proteins**

15   In vitro systems may be designed to identify compounds
capable of binding the target proteins of the invention.
Compounds identified may be useful, for example, in
modulating the activity of wild type and/or mutant target
proteins, may be useful in elaborating the biological

20   function of the target protein, may be utilised in
screens for identifying compounds that disrupt normal
target protein interactions, or may in themselves disrupt
such interactions.

25   The principle of the assays used to identify compounds
that bind to the target protein involves preparing a
reaction mixture of the target protein and the test
compound under conditions and for a time sufficient to
allow the two components to interact and bind, thus

30   forming a complex which can be removed and/or detected in
the reaction mixture. These assays can be conducted in a
variety of ways. For example, one method to conduct such
an assay would involve anchoring target protein or the

test substance onto a solid phase and detecting target
protein/test compounds complexes anchored on the solid
phase at the end of the reaction. In one embodiment of
such a method, the target protein may be anchored onto a
5   solid surface, and the test compound, which is not
anchored, may be labelled, either directly or indirectly.

In practice, microtiter plates may conveniently be
utilised as the solid phase. The anchored component may
10   be immobilised by non-covalent or covalent attachments.
Non-covalent attachment may be accomplished by simply
coating the solid surface with a solution of the protein
and drying. Alternatively, an immobilised antibody,
preferably a monoclonal antibody, specific for the
15   protein to be immobilised may be used to anchor the
protein to the solid surface. The surfaces may be
prepared in advance and stored.

In order to conduct the assay, the non-immobilised
20   component is added to the coated surface containing the
anchored component. After the reaction is complete,
unreacted components are removed (e.g. by washing) under
conditions such that any complexes formed will remain
immobilised on the solid surface. The detection of
25   complexes anchored on the solid surface can be
accomplished in a number of ways. Where the previously
non-immobilised component is pre-labelled, the detection
of label immobilised on the surface indicates that
complexes were formed. Where the previously non-
30   immobilised component is not pre-labelled, an indirect
label can be used to detect complexes anchored on the
surface, e.g. using a labelled antibody specific for the
previously non-immobilised component (the antibody, in

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turn, may be directly labelled or indirectly labelled
with a labelled anti-Ig antibody).

Alternatively, a reaction can be conducted in a liquid
5   phase, the reaction products separated from unreacted
components, and complexes detected, e.g. using an
immobilised antibody specific for target protein or the
test compound to anchor any complexes formed in solution,
and a labelled antibody specific for the other component
10   of the possible complex to detect anchored complexes.

**3.2 Assays for cellular proteins that interact with the
target protein**
Any method suitable for detecting protein-protein
15   interactions may be employed for identifying novel target
protein-cellular or extracellular protein interactions.
These methods are outlined in Section 1.2, above, for the
identification of pathway proteins, and may be utilised
herein with respect to the identification of proteins
20   which interact with identified target proteins.

**3.3 Assays for compounds that interfere with target
protein/cellular macromolecule interaction**
The target proteins of the invention may, in vivo,
25   interact with one or more cellular or extracellular
macromolecules, such as proteins. Such macromolecules
may include, but are not limited to, nucleic acid
molecules and those proteins identified via methods such
as those described above, in Section 3.2. For purposes
30   of this discussion, such cellular and extracellular
macromolecules are referred to herein as 'binding
partners'. Compounds that disrupt such interactions may
be useful in regulating the activity of the target

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protein, especially mutant target proteins. Such
compounds may include, but are not limited to, molecules
such as antibodies, peptides, and the like, as described,
for example, in Section 3.1 above.

5

The basic principle of the assay systems used to identify
compounds that interfere with the interaction between the
target protein and its cellular or extracellular binding
partner or partners involves preparing a reaction mixture
10   containing the target protein, and the binding partner
under conditions and for a time sufficient to allow the
two to interact and bind, thus forming a complex. In
order to test a compound for inhibitory activity, the
reaction mixture is prepared in the presence and absence
15   of the test compound. The test compound may be initially
included in the reaction mixture, or may be added at a
time subsequent to the addition of target protein and its
cellular or extracellular binding partner. Control
reaction mixtures are incubated without the test compound
20   or with a placebo. The formation of any complexes
between the target protein and the cellular or
extracellular binding partner is then detected. The
formation of a complex in the control reaction, but not
in the reaction mixture containing the test compound
25   indicates that the compound interferes with the
interaction of the target protein and the interactive
binding partner. Additionally, complex formation within
reaction mixtures contains the test compound and a mutant
target protein. This comparison may be important in
30   those cases wherein it is desirable to identify compounds
that disrupt interactions of mutant but not normal target
proteins.

The assay for compounds that interfere with the
interaction of the target and binding partners can be
conducted in a heterogeneous or homogeneous format.
Heterogeneous assays involve anchoring either the target
5   protein or the binding partner onto a solid phase and
detecting complexes anchored on the solid phase at the
end of the reaction. In homogeneous assays, the entire
reaction is carried out in a liquid phase. In either
approach, the order of addition of reactants can be
10   varied to obtain different information about the
compounds being tested. For example, test compounds that
interfere with the interaction between the target protein
and the binding partners, e.g. by competition, can be
identified by conducting the reaction in the presence of
15   the test substance, i.e. by adding the test substance to
the reaction mixture prior to or simultaneously with the
target protein and interactive cellular or extracellular
binding partner. Alternatively, test compounds that
disrupt pre-formed complexes, e.g. compounds with higher
20   binding constants that displace one of the components
from the complex, can be tested by adding the test
compound to the reaction mixture after complexes have
been formed. The various formats are described briefly
below.

25

In a heterogeneous assay system, either the target
protein or the interactive cellular or extracellular
binding partner, is anchored onto a solid surface, while
the non-anchored species is labelled, either directly or
30   indirectly. In practice, microtiter plates are
conveniently utilised. The anchored species may be
immobilised by non-covalent or covalent attachments.
Non-covalent attachment may be accomplished simply by

coating the solid surface with a solution of the target
gene product or binding partner and drying.
Alternatively, an immobilised antibody specific for the
species to be anchored may be used to anchor the species
5   to the solid surface. The surfaces may be prepared in
advance and stored.

In order to conduct the assay, the partner of the
immobilised species is exposed to the coated surface with
10   or without the test compound. After the reaction is
complete, unreacted components are removed (e.g. by
washing) and any complexes formed will remain immobilised.
on the solid surface. The detection of complexes
anchored on the solid surface can be accomplished in a
15   number of ways. Where the non-immobilised species is
pre-labelled, the detection of label immobilised on the
surface indicates that complexes were formed. Where the
non-immobilised species is not pre-labelled, an indirect
label can be used to detect complexes anchored on the
20   surface, e.g. using a labelled antibody specific for the
initially non-immobilised species (the antibody, in turn,
may be directly labelled or indirectly labelled with a
labelled anti-Ig antibody). Depending upon the order of
addition of reaction components, test compounds which
25   inhibit complex formation or which disrupt pre-formed
complexes can be detected.

Alternatively, the reaction can be conducted in a liquid
phase in the presence or absence of the test compound,
30   the reaction products separated from unreacted
components, and complexes detected, e.g. using an
immobilised antibody specific for one of the binding
components to anchor any complexes formed in solution,

and a labelled antibody specific for the other partner to
detect anchored complexes. Again, depending upon the
order of addition of reactants to the liquid phase, test
compounds which inhibit complex or which disrupt pre-
5   formed complexes can be identified.

In an alternate embodiment of the invention, a
homogeneous assay can be used. In this approach, a pre-
formed complex of the target protein and the interactive
10   cellular or extracellular binding partner is prepared in
which either the target protein or its binding partners
is labelled, but the signal generated by the label is
quenched due to complex formation (see, e.g. US Pat. No.
4,109,496 by Rubenstein which utilises this approach for
15   immunoassays). The addition of a test substance that
competes with and displaces one of the species from the
pre-formed complex will result in the generation of a
signal above background. In this way, test substances,
which disrupt target protein/cellular or extracellular
20   binding partner interaction, can be identified.

In a particular embodiment, the target protein can be
prepared for immobilisation using recombinant DNA
techniques described in Section 2.1 above. For example,
25   the target protein gene coding region can be fused to a
glutathione-S-transferase (GST) gene using a fusion
vector, such as pGEX-5X-1, in such a manner that its
binding activity is maintained in the resulting fusion
protein. The interactive cellular or extracellular
30   binding partner can be purified and used to raise a
monoclonal antibody, using methods routinely practised in
the art and described above, in Section 2.1. This
antibody can be labelled with the radioactive isotope

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^{125}I, for example, by methods routinely practised in the
art.   In a heterogeneous assay, e.g. the GST-target
protein gene fusion protein can be anchored to
glutathione-agarose beads.   The interactive cellular or
5   extracellular binding partner can then be added in the
presence or absence of the test compound in a manner that
allows interaction and binding to occur.   At the end of
the reaction period, unbound material can be washed away,
and the labelled monoclonal antibody can be added to the
10   system and allowed to bind to the complexed components.
The interaction between the target protein and the
interactive cellular or extracellular binding partner can
be detected by measuring the amount of radioactivity that
remains associated with the glutathione-agarose beads.   A
15   successful inhibition of the interaction by the test
compound will result in a decrease in measured
radioactivity.

Alternatively, the GST-target protein gene fusion protein
20   and the interactive cellular or extracellular binding
partner can be mixed together in liquid in the absence of
the solid glutathione-agarose beads.   The test compound
can be added either during or after the species are
allowed to interact.   This mixture can then be added to
25   the glutathione-agarose beads and unbound material is
washed away.   Again the extent of inhibition of the
target protein/binding partner interaction can be
detected by adding the labelled antibody and measuring
the radioactivity associated with the beads.

30
In another embodiment of the invention, these same
techniques can be employed using peptide fragments that
correspond to the binding domains of the target protein

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and/or the interactive cellular or extracellular binding
partner (in cases where the binding partner is a
protein), in place of one or both of the full length
proteins. Any number of methods routinely practised in
5   the art can be used to identify and isolate the binding
sites. These methods include, but are not limited to,
mutagenesis of the gene encoding one of the proteins and
screening for disruption of binding in a co-
immunoprecipitation assay. Compensating mutations in the
10   gene encoding the second species in the complex can then
be selected. Sequence analysis of the genes encoding the
respective proteins will reveal the mutations that
correspond to the region of the protein involved in
interactive binding. Alternatively, one protein can be
15   anchored to a solid surface using methods described in
this Section above, and allowed to interact with and bind
to its labelled binding partner, which has been treated
with a proteolytic enzyme, such as trypsin. After
washing, a short, labelled peptide comprising the binding
20   domain may remain associated with the solid material,
which can be isolated and identified by amino acid
sequencing. Also, once the gene coding for the cellular
or extracellular binding partner is obtained, short gene
segments can be engineered to express peptide fragments
25   of the protein, which can then be tested for binding
activity and purified or synthesised.

For example, and not by way of limitation, a target
protein can be anchored to a solid material as described
30   above, in this Section by making a GST-target protein
gene fusion protein and allowing it to bind to
glutathione agarose beads. The interactive cellular or
extracellular binding partner can be labelled with a

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radioactive isotope, such as ^{35}S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-target protein gene fusion protein and allowed to bind. After washing away

5   unbound peptides, labelled bound material, representing the cellular or extracellular binding partner binding domain, can be eluted, purified and analysed for amino acid sequence by well-known methods. Peptides so identified can be produced synthetically or fused to

10   appropriate facilitative proteins using recombinant DNA technology.

3.4   Assays for amelioration of body weight and/or eating disorder symptoms

15   Any of the binding compounds, including but not limited to, compounds such as those identified in the foregoing assay systems, may be tested for the ability to prevent or ameliorate body weight and/or eating disorder symptoms, which may include, for example, obesity. Cell-

20   based and animal model-based assays for the identification of compounds exhibiting such an ability to ameliorate body weight and/or eating disorder symptoms are described below.

25   First, cell-based systems such as those described above, in Section 2.2.2, may be used to identify compounds, which may act to prevent or ameliorate body weight and/or eating disorder symptoms. For example, such cell systems may be exposed to a compound, suspected of exhibiting an

30   ability to ameliorate body weight and/or eating disorder symptoms, at a sufficient concentration and for a time sufficient to elicit a response in the exposed cells. After exposure, the cells are examined to determine

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whether one or more of the body weight and/or eating
disorder-like cellular phenotypes has been altered to
resemble a more normal or more wild type phenotype, or a
phenotype more likely to produce a lower incidence or
5   severity of disorder symptoms.

In addition, animals-based body weight and/or eating
disorder systems, such as those described above, in
Section 2.2.1, may be used to identify compounds capable
10   of ameliorating body weight and/or eating disorder-like
symptoms. Such animal models may be used as test
substrates for the identification of drugs,
pharmaceuticals, therapies and interventions which may be
effective in treating such disorders. For example,
15   animal models may be exposed to a compound, suspected of
exhibiting an ability to prevent or ameliorate body
weight and/or eating disorder symptoms, at a sufficient
concentration and for a time sufficient to elicit such a
prevention or amelioration of the body weight and/or
20   eating disorder symptoms in the exposed animals. The
response of the animals to the exposure may be monitored
by assessing the reversal of disorders associated with
body weight and/or eating disorders such as obesity.

25   With regard to intervention, any treatments that reverse
any aspect of body weight and/or eating disorder-like
symptoms should be considered as candidates for human or
companion animal body weight and/or eating disorder
therapeutic intervention including the treatment of
30   obesity. Dosages of test agents may be determined by
deriving dose-response curves, as discussed in Section
6.1 below.

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Similarly any treatments that can prevent the development
or redevelopment of body weight and/or eating disorders
should be considered as candidates for the prevention of
human or companion animal body weight and/or eating
5   disorder therapeutic intervention. Such disorders
include, but are not limited to, obesity.

Protein expression patterns may be utilised in
conjunction with either cell-based or animal-based
10   systems to assess the ability of a compound to ameliorate
body weight and/or eating disorder-like symptoms. For
example, the expression pattern of one or more
fingerprint proteins may form part of a fingerprint
profile, which may then be used in such as assessment.
15   Fingerprint profiles are described below, in Section 7.1.
Fingerprint profiles may be characterised for known
states, either body weight and/or eating disorder or
normal states, within the cell- and/or animal-based model
systems. Subsequently, these known fingerprint profiles
20   may be compared to ascertain the effect a test compound
has to modify such fingerprint profiles, and to cause the
profile to more closely resemble that of a more desirable
fingerprint. For example, administration of a compound
may cause the fingerprint profile of a body weight and/or
25   eating disorder model system to more closely resemble the
control system. Administration of a compound may,
 alternatively, cause the fingerprint profile of a control
system to begin to mimic a body weight and/or eating
disorder state, which may, for example, be used in
30   further characterising the compound of interest, or may
be used in the generation of additional animal models.

4.   Compounds and methods for treatment of body weight

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<u>and/or eating disorders</u>

Described below are methods and compositions whereby body
weight and/or eating disorder symptoms may be
ameliorated. It is possible that body weight and/or

5   eating disorders may be brought about, at least in part,
by an abnormal level of target protein, or by the
presence of a target protein exhibiting an abnormal
activity. As such, the reduction in the level and/or
activity of such target protein would bring about the

10   amelioration of body weight and/or eating disorder-like
symptoms. Techniques for the reduction of target protein
gene expression levels or target protein activity levels
are discussed in Section 4.1 below.

15   Alternatively, it is possible that body weight and/or
eating disorders may be brought about, at least in part,
by the absence or reduction of the level of target
protein expression, or a reduction in the level of a
target proteins activity. As such, an increase in the

20   level of target protein gene expression and/or the
activity of such proteins would bring about the
amelioration of body weight and/or eating disorder-like
symptoms. Techniques for increasing target protein gene
expression levels or target protein activity levels are

25   discussed in Section 4.2 below.

**4.1 <u>Compounds that inhibit expression, synthesis or
activity of mutant target proteins</u>**

As discussed above, target proteins involved in body

30   weight and/or eating disorders may cause such disorders
via an increased level of target protein activity. A
variety of techniques may be utilised to inhibit the
expression, synthesis, or activity of such target genes

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and/or proteins.

For example, compounds such as those identified through
assays described above, in Section 3, which exhibit

5   inhibitory activity, may be used in accordance with the
invention to prevent or ameliorate body weight and/or
eating disorder symptoms. As discussed in Section 3
above, such molecules may include, but are not limited
to, peptides (such as, for example, peptides representing

10   soluble extracellular portions of target protein
transmembrane receptors), phosphopeptides, small organic
or inorganic molecules, or antibodies (including, for
example, polyclonal, monoclonal, humanised, anti-
idiotypic, chimeric or single chain antibodies, and FAb,

15   F(ab')$_2$ and FAb expression library fragments, and epitope-
binding fragments thereof). Techniques for determination
of effective doses and administration of such compounds
are described below, in Section 6.1. Inhibitory antibody
techniques are further described below, in Section 4.1.2.

20

Further, antisense and ribosome molecules, which inhibit
expression of the target protein gene, may also be used
in accordance with the invention to inhibit the aberrant
target protein gene activity. Such techniques are

25   described below, in Section 4.1.1; triple helix molecules
may be utilised in inhibiting the aberrant target protein
gene activity.

4.1.1   Inhibitory antisense, ribosome and triple helix
30   **approaches**
Among the compounds, which may exhibit the ability to
prevent or ameliorate body weight and/or eating disorder
symptoms are antisense, ribosome and triple helix

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molecules. Such molecules may be designed to reduce or
inhibit either wild type, or if appropriate, mutant
target protein gene activity. Techniques for the
production and use of such molecules are well known to
5   those of skill in the art.

Antisense RNA and DNA molecules act to directly block the
translation of mRNA by hybridising to targeted mRNA and
preventing protein translation. With respect to
10   antisense DNA, oligodeoxy-ribonucleotides derived from
the translation initiation site, e.g. between the -10 and
+10 regions of the target gene nucleotide sequence of
interest, are preferred.

15   Ribosomes are enzymatic RNA molecules capable of
catalysing the specific cleavage of RNA. (For a review,
see Rossi, J., 1994, Current Biology 4: 469-471). The
mechanism of ribosome action involves sequence specific
hybridisation of the ribosome molecule to complementary
20   target RNA, followed by an endonucleolytic cleavage. The
composition of ribosome molecules must include one or
more sequences complementary to the target protein mRNA,
and must include the well known catalytic sequence
responsible for mRNA cleavage. For this sequence, see US
25   Pat. No. 5,093,246, which is incorporated by reference
herein in its entirety. As such, within the scope of the
invention are engineered hammerhead motif ribosome
molecules that specifically and efficiently catalyse
endonucleolytic cleavage of RNA sequences encoding target
30   proteins.

Specific ribosome cleavage sites within any potential RNA
target are initially identified by scanning the molecule

of interest for ribosome cleavage sites which include the
following sequences, GUA, GUU and GUC. Once identified,
short TNA sequences of between 15 and 20 ribonucleotides
corresponding to the region of the target protein gene,

5   containing the cleavage site may be evaluated for
predicted structural features, such as secondary
structure, that may render the oligonucleotide sequence
unsuitable. The suitability of candidate sequences may
also be evaluated by testing their accessibility to

10   hybridise with complementary oligonucleotides, using
ribonuclease protection assays.

Nucleic acid molecules to be used in triplex helix
formation for the inhibition of transcription should be

15   single stranded and composed of deoxynucleotides. The
base composition of these oligonucleotides must be
designed to promote triple helix formation via Hoogsteen
base pairing rules, which generally require sizeable
stretches of either purines or pyrimidines to be present

20   on one strand of a duplex. Nucleotide sequences may be
pyrimidine-based, which will result in TAT and CGC⁺
triplets across the three associated strands of the
resulting triple helix. The pyrimidine-rich molecules
provide base complementary to a purine-rich region of a

25   single strand of the duplex in a parallel orientation to
that strand. In addition, nucleic acid molecules may be
chosen that are purine-rich, for example, containing a
stretch of G residues. These molecules will form a
triple helix with a DNA duplex that is rich in GC pairs,

30   in which the majority of the purine residues are located
on a single strand of the targeted duplex, resulting in
GGC triplets across the three strands in the triplex.

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Alternatively, the potential sequences that can be
targeted for triple helix formation may be increased by
creating a so-called "switchback" nucleic acid molecule.
Switchback molecules are synthesised in an alternating

5   5'-3', 3'-5' manner, such that they base pair with first
one strand of a duplex and then the other, eliminating
the necessity for a sizeable stretch of either purines or
pyrimidines to be present on one strand of a duplex.

10   Anti-sense RNA and DNA, ribosome and triple helix
       molecules of the invention may be prepared by any method
       known in the art for the synthesis of DNA and RNA
       molecules. They include techniques for chemically
       synthesising oligodeoxyribonucleotides and oligo-
15   ribonucleotides well known in the art such as, for
       example, solid phase phosphoramidite chemical synthesis.
       Alternatively, RNA molecules may be generated by in vitro
       and in vivo transcription of DNA sequences encoding the
       antisense RNA molecule. Such DNA sequences may be
20   incorporated into a wide variety of vectors, which
       incorporate suitable RNA polymerase promoters such as the
       T7 or SP6 polymerase promoters. Alternatively, antisense
       cDNA constructs that synthesise antisense RNA
       constitutively inducibly, depending on the promoter used,
25   can be introduced stably into cell lines.

       Various well-known modifications to the DNA molecules may
       be introduced as a means of increasing intracellular
       stability and half-life. Possible modifications include,
30   but are not limited to, the addition of flanking
       sequences or ribo- or deoxy-nucleotides to the 5' and/ or
       3' ends of the molecule or the use of phosphorothioate or
       2' O-methyl rather than phosphodiesterase linkages within

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the oligodeoxy-ribonucleotide backbone.

4.1.2   Antibodies for the inhibition of target protein

Antibodies that are both specific for target protein and

5   interfere with its activity may be used to inhibit target

protein function. Where desirable, antibodies specific

for mutant target protein, which interferes with the

activity of such mutant target product, may also be used.

Such antibodies may be generated using standard

10   techniques described in Section 2.3, supra, against the

proteins themselves or against peptides corresponding to

portions of the proteins. The antibodies include, but

are not limited to, polyclonal, monoclonal, Fab

fragments, single chain antibodies, chimeric antibodies,

15   etc.

In instances where the target gene protein is

intracellular and whole antibodies are used,

internalising antibodies may be preferred. However,

20   lipofectin or liposomes may be used to deliver the

antibody or a fragment of the Fab region, which binds to

the target protein epitope into cells. Where fragments

of the antibody are used, the smallest inhibitory

fragment, which binds to the target protein's binding

25   domain, is preferred. For example, peptides having an

amino acid sequence corresponding to the domain of the

variable region of the antibody that binds to the target

protein may be used. Such peptides may be synthesised

chemically or produced via recombinant DNA technology

30   using methods well known in the art (e.g. see Creighton,

1983, supra; and Sambrook et al, 1989, supra).

Alternatively, single chain neutralising antibodies,

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which bind to intracellular target protein epitopes, may
also be administered. Such single chain antibodies may
be administered, for example, by expressing nucleotide
sequences encoding single-chain antibodies within the

5   target cell populating by utilising, for example,
techniques such as those described in Marasco et al
(Marasco, W. et al, 1993, Proc. Natl. Acad. Sci. USA, 90:
7889-7893).

10   In instances where the target protein is extracellular,
or is a transmembrane protein, any of the administration
techniques described below, in Section 6, which are
appropriate for peptide administration may be utilised to
effectively administer inhibitory target protein

15   antibodies to their site of action.

        4.2 Methods for restoring target protein activity
        Target proteins that cause body weight and/or eating
        disorders may be underexpressed within body weight and/or

20   eating disorder situations. Alternatively, the activity
        of target protein may be diminished, leading to the
        development of body weight and/or eating disorder
        symptoms. Described in this Section are methods whereby
        the level of target protein may be increased to levels

25   wherein body weight and/or eating disorder symptoms are
        prevented or ameliorated. The level of target protein
        activity may be increased, for example, by either
        increasing the level of target protein present or by
        increasing the level of active target protein, which is

30   present.

        For example, a target protein, at a level sufficient to
        ameliorate body weight and/or eating disorder symptoms

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may be administered to a patient exhibiting such
symptoms. Any of the techniques discussed below, in
Section 6, may be utilised for such administration. One
of skill in the art will readily know how to determine
the concentration of effective, non-toxic doses of the
normal target protein, utilising techniques such as those
described below, in Section 4.6.1.

Further, patients may be treated by gene replacement
therapy. One or more copies of a normal target protein
gene or a portion of the gene that directs the production
of a normal target protein with target protein gene
function, may be inserted into cells, using vectors which
include, but are not limited to, adenovirus, adeno-
associated virus, and retrovirus vectors, in addition to
other particles that introduce DNA into cells, such as
liposomes. Additionally, techniques such as those
described above may be utilised for the introduction of
normal target protein gene sequences into human cells.

Cells, preferably autologous cells, containing normal
target protein gene sequences may then be introduced or
reintroduced into the patient at positions which allow
for the prevention or amelioration of body weight and/or
eating disorder symptoms. Such cell replacement
techniques may be preferred, for example, when the target
protein is a secreted, extracellular protein.

Additionally, antibodies may be administered which
specifically bind to a target protein and by binding,
serve to, either directly or indirectly, activate the
target protein function. Such antibodies can include,
but are not limited to, polyclonal, monoclonal, FAb

fragments, single chain antibodies, chimeric antibodies and the like. The antibodies may be generated using standard techniques such as those described above, in Section 2.3, and may be generated against the protein themselves or against proteins corresponding to portions of the proteins. The antibodies may be administered, for example, according to the techniques described above, in Section 4.1.2.

5.   Pharmaceutical preparations and methods of administration

The identified compounds, nucleic acid molecules and cells that affect target protein expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to prevent or to treat or to ameliorate body weight and/or eating disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of body weight and/or eating disorder, including obesity, or alternatively, to that amount of a nucleic acid molecule sufficient to express a concentration of protein which results in the amelioration of such symptoms.

5.1   Effective dose

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g. for determining by ED_{50} (the dose therapeutically effective in 50% of the population) and by determining the ED_{50} of any side-effects (toxicity - TD50). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio TD_{50}/ED_{50}. Compounds,

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which exhibit large therapeutic indices, are preferred.
While compounds that exhibit toxic side effects may be
used, care should be taken to design a delivery system
that targets such compounds to the site of affected
5   tissue in order to minimise potential damage to
uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal
studies can be used in formulating a range of dosage for
10   use in humans. The dosage of such compounds lies
preferably within a range of circulating concentrations
that include the ED_{50} with little or no toxicity. The
dosage may vary within this range depending upon the
dosage form employed and the route of administration
15   utilised.

5.2   Formulations and use

Pharmaceutical compositions for use in accordance with
the present invention may be formulated in conventional
20   manner using one or more physiologically acceptable
carriers or excipients.

Thus, the compounds and their physiologically acceptable
salts and solvates may be formulated for administration
25   by inhalation or insufflation (either through the mouth
or the nose) or oral, buccal, parenteral and rectal
administration.

For oral administration, the pharmaceutical compositions
30   may take the form of, for example, tablets or capsules
prepared by conventional means with pharmaceutically
acceptable excipients such as binding agents (e.g. pre-
gelatinised maize starch, polyvinylpyrrolidone or

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hydroxypropyl methyl-cellulose); fillers (e.g. lactose, microcrystaklline cellulose or calcium hydrogen phosphate); lubricants (e.g. magnesium, stearate, talc or silica); disintegrants (e.g. potato starch or sodium

5   starch glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be

10   presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, cellulose derivatives or

15   hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavouring, colouring and sweetening agents as appropriate.

20

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

25   For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use
30   according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane,

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trichlorofluoromethane, dichloro-tetrafluoroethane,
carbon dioxide or other suitable gas. In the case of a
pressurised aerosol the dosage unit may be determined by
providing a valve to deliver a metered amount. Capsules

5   and cartridges of, e.g. gelatin, for use in an inhaler or
insufflator may be formulated containing a powder mix of
the compound and a suitable powder base such as lactose
or starch.

10   The compounds may be formulated for parenteral
administration by injection, e.g. by bolus injection or
continuous infusion. Formulations for injection may be
presented in unit dosage form, e.g. in ampoules or in
multi-dose containers, with an added preservative. The

15   compositions may take such forms as suspensions,
solutions or emulsions in oily or aqueous vehicles, and
may contain formulatory agents such as suspending,
stabilising and/or dispersing agents. Alternatively, the
active ingredient may be in powder form for constitution

20   with a suitable vehicle, e.g. sterile pyrogen-free water,
before use.

The compounds may also be formulated in rectal
compositions such as suppositories or retention enemas,

25   e.g. containing conventional suppository bases such as
cocoa butter or other glycerides.

In addition to the formulations described previously, the
compounds may also be formulated as a depot preparation.

30   Such long acting formulations may be administered by
implantation, for example, subcutaneously or
intramuscularly) or by intramuscular injection. Thus,
for example, the compounds may be formulated with

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suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as blister pack. The pack or dispenser device may be accompanied by instructions for administration.

6.   Diagnosis of the nature of body weight and/or eating disorder abnormalities including obesity

A variety of methods may be employed for the diagnosis of the nature of the body weight and/or eating disorders, the predisposition to body weight and/or eating disorders, and for monitoring the efficacy of any body weight and/or eating disorder compounds during, for example, clinical trials and for monitoring patients undergoing clinical evaluation for the treatment of such disorders.

The methods include conventional methods such as waist/hip ratios (BMI), weighing and c.t. or dexascans.

Obesity may be detected and the efficacy of treatment monitored by methods and parameters identified by such bodies as the World Health Organisation and the International Obesity Task Force.

Methods may also, for example, utilise reagents such as the fingerprint protein described in Section 4.1, and

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antibodies directed against differentially expressed and
pathway proteins, as described above, in Sections 1.3
(peptides) and 2.3 (antibodies). Specifically, such
reagents may be used, for example, for: (1) the

5   detection of the presence of target protein mutations, or
(2) the detection of either an over- or an under-
abundance of target protein relative to the normal state.

The methods described herein may be performed, for

10   example, by utilising pre-packaged diagnostic kits
comprising at least one specific finger print protein or
anti-fingerprint protein antibody reagent described
herein, which may be conveniently used, e.g. in clinical
settings, to diagnose patients exhibiting body weight

15   and/or eating abnormalities.

Any cell type or tissue in which the fingerprint protein
is expressed may be utilised in the diagnostics described
below. Examples of suitable samples types include cell

20   samples, tissue samples, and fluid samples such as blood,
urine or plasma.

Among the methods, which can be utilised herein, are
methods for monitoring the efficacy of compounds in

25   clinical trials for the treatment of body weight and/or
eating disorders. Such compounds can, for example, be
compounds such as those described above, in Section 4.
Such a method comprises detecting, in a patient sample, a
protein, which is differentially expressed in the body

30   weight and/or eating disorder state relative to its
expression in a normal state.

During clinical trials, for example, the expression of a

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single fingerprint protein, or alternatively, a
fingerprint pattern of a cell involved in a body weight
and/or eating disorder can be determined in the presence
or absence of the compound being tested. The efficacy of
5   the compound can be followed by comparing the expression
data obtained to the corresponding known expression
patterns in a normal state. Compounds exhibiting
efficacy are those which alter the single fingerprint
protein expression and/or the fingerprint pattern to more
10   closely resemble that of the normal state.

The detection of the protein differentially expressed in
a body weight and/or eating disorder state relative to
their expression in a normal state can also be used for
15   monitoring the efficacy of potential body weight and/or
eating disorder compounds and compounds for the treatment
of obesity during clinical trials. During clinical
trials, for example, the level and/or activity of the
differentially expressed protein can be determined in
20   relevant cells and/or tissues in the presence or absence
of the compound being tested. The efficacy of the
compound can be followed by comparing the protein level
and/or activity data obtained to the corresponding known
levels/activities for the cells and/or tissues in a
25   normal state. Compounds exhibiting efficacy are those
which alter the pattern of the cell and/or tissue
involved in the body weight and/or eating disorder to
more closely resemble that of the normal state.

30   **EXAMPLE 1**

Mouse treatment protocol
Non-fasted lean and obese, 8 week old, female C57 B1/6J
ob/ob mice were anaesthetised with 50% "Hyponovel" and

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50% "Hyponorm" and then killed humanely with carbon
dioxide gas. Liver was removed and snap frozen between
tongs in liquid nitrogen. The tissues were lyophilised
for 48h, crushed in a mortar with liquid nitrogen and the
5   resultant dried powder stored at -80°C

Protein solubilisation

For analytical 2-D-PAGE, 200 micrograms of dried liver
was mixed with 60 microlitres of a solution containing
10   urea (8M), CHAPS (4% w/v), Tris (40mM), DTE (65mM), SDS
(0.05% w/v) and a trace of bromophenol blue. A weighed
portion of the final diluted sample was loaded into a
sample cup at the cathodic end of the IPG gels.

15   **First dimension electrophoresis**

A non-linear immobilised pH gradient of IGP strips (3.5-
10.0 NL IPG 18cm) was used as the first dimension. It
offered high resolution, great reproducibility and
allowed high protein loads. Based on specifications of
20   the Geneva University Hospital, the non-linear pH
gradient strips were prepared by Amersham-Pharmacia
Biotechnology AB and are commercially available. The
strips were 3mm wide and 180mm long.

25   Hydration of the IPG strips was performed overnight in a
Pharmacia reswelling cassette with 25ml of a solution of
urea (8M), CHAPS (2% w/v), DTE (10mM), Resolyte pH 3.5-10
(2% v/v) and a trace of bromophenol blue.

30   When the rehydration cassette had been thoroughly emptied
and opened, the strips were transferred to the Pharmacia
strip tray. After placing IPG strips, humid electrode
wicks, electrodes and sample cups in position, the strips

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and cups were covered with low viscosity paraffin oil.
Samples were applied in the cups at the cathodic end of
the IPG strips in a slow and continuous manner, without
touching the gel.

5

The voltage was linearly increased from 300 to 3500 V
during 3 hours, followed by 3 additional hours at 3500 V,
whereupon the voltage was increased to 5000 V. A total
volt hour product of 100kvh was used in an overnight run.

10

Second dimension of the electrophoresis

After the first dimension run, the IPG strips were
equilibrated in order to resolubilise the proteins and to
reduce -S-S- bonds. The strips were thus equilibrated

15   within the strip tray with 100ml of a solution containing
Tris-HCl (50mM), pH 6.8, urea (6M), glycerol (30% v/v),
SDS (2% w/v) and DTE (2% w/v) for 12 min. The SH groups
were subsequently blocked with 100 ml of a solution
containing Tris-HCl (50mM) pH 6.8, urea (6M), glycerol

20   (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a
trace of bromophenol blue for 5 min.

In the second dimension run, a vertical gradient slab gel
with the Laemmli-SDS-discontinuous system was used with

25   some small modifications, which may be summarised as
follows:

•   Gels are not polymerised in the presence of SDS.
This seems to prevent the formation of micelles,

30   which contain acrylamide monomer, thus increasing
the homogeneity of pore size and reducing the
concentration of unpolymerised monomer in the
polyacrylamide. The SDS used in the gel running

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buffer is sufficient to maintain the necessary
negative charge on proteins.

- Piperazine-diacrylyl (PDA) is used as crosslinker.
  This is believed to reduce N-terminal protein
  blockage, gives better protein resolution, and
  reduces diammine silver staining background.

- Sodium thiosulphate is used as an additive to reduce
  background in the silver staining of gels.

- The combination of the IPG strip and agarose avoids
  the need for a stacking gel.

The gel composition and dimensions were as follows:

Dimensions:	160 x 200 x 1.5mm
Resolving gel:	Acrylamide/PDA (9-16%
T/2.6% C)	
Stacking gel:	No stacking
Leading buffer:	Tris-HCl (0.375M) pH 8.8
Trailing buffer:	Tris-glycine-SDS (25mM-
198mM-0.1% w/v) pH 8.3	
Additives:	Sodium thiosulphate (5mM)
Polymerisation agents:	TEMED (0.05%) APS (0.1%)

The gels were poured until 0.7cm from the top of the
plates and over-layered with sec-butanol for about two
hours. After the removal of the overlay and its
replacement with water the gels were left overnight.

After the equilibration, the IPG gel strips were cut to
size. Six mm were removed from the anodic end and 14mm
from the cathodic end. The second dimension gels were

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over-layered with a solution containing agarose (0.5%
w/v) and Tris-glycine-SDS (25mM-198mM-0.1% w/v) pH 8.3
heated at about 70°C and the IPG gel strips were
immediately loaded through it.

The gel was run at 8-12°C for 5 hours at a constant
current of 40mA/gel. The voltage is non-limiting, but
usually requires 100 to 400 V.

Staining

Silver staining, which is 100-fold more sensitive that
Coomassie Brilliant Blue staining, was used (except where
otherwise stated). Thus, the 2-DGE gels were stained
with an ammoniacal silver staining as follows:

All steps were performed on an orbital shaker at 36 rpm.
Step 1:   At the end of the second dimension run, the
gels were removed from the glass plates and washed in
deionised water for 5 min.
Step 2:   The gels were soaked in ethanol:acetic
acid:water (40:10:50 volume ratio) for 1 hour.
Step 3:   The gels were soaked in ethanol:acetic
acid:water (5:5:90 volume ratio) for 2 hours or
overnight.
Step 4:   They were washed in deionised water for 5 min.
Step 5:   They were soaked in a solution containing
glutaraldehyde (1% w/v and sodium acetate (0.5M) for 30
min.
Step 6:   They were washed 3 times in deionised water for
10 min.
Step 7:   In order to obtain homogenous dark brown
staining of proteins, gels were soaked twice in a 2, 7-
naphthalenedisulphonic acid solution (0.05% w/v) for 30

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min.

Step 8:   The gels were then rinsed 4 times in deionised water for 15 min.

Step 9:   The gels were stained in a freshly made

5   ammoniacal silver nitrate solution for 30 minutes.   To prepare 750ml of this solution, 6g of silver nitrate were dissolved in 30ml of deionised water, which was slowly mixed into a solution containing 160ml of water, 10ml of concentrated ammonia (25%) and 1.5ml o sodium hydroxide

10   (10N).   A transient brown precipitate might form.   After it cleared, water was added to give the final volume.

Step 10:   After staining, the gels were washed 4 times in deionised water for 4 min.

Step 11:   The images were developed in a solution of

15   citric acid (0.01% w/v) and formaldehyde (0.1% v/v) for 5 to 10 min.

Step 12:   When a slight background stain appeared, development was stopped with a solution of Tris (5% w/v) and acetic acid (2% v/v).

20

Scanning of the gels

The Laser Densitometer (4000 x 5000 pixels; 12 bits/pixel) from Molecular Dynamics and the GS-700 from Bio-Rad have been used as scanning devices.   These

25   scanners were linked to "Sparc" workstations and "Macintosh" computers.

Quantitative image analysis of the gels using "Melanie II"

30   Two-dimensional polyacrylamide gels may be digitised and analysed by computer to allow quantitative image analysis and automatic gel comparison.   Since the 2-D-PAGE technique was first developed in 1975 several computer

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systems have been manufactured, mainly by academic 2-D-
PAGE related laboratories.   In the present work, "Melanie
II", developed at the University Hospital of Geneva was
used.   It is available for "Unix" workstations, as well
5   as for "Power Macintosh" and IBM-compatible computers.

ob/ob and lean mice spot detection, quantitation and
matching, gel image extraction, zooming, warping and
printing as well as gel stacking and flipping were
10   carried out with the "MelView" program.

The following DEPs were found which were all underexposed
in ob/ob mice relative to expression in lean mice.

                    LOM 16
15   LOM 17
                    LOM 18
                    LOM 19
                    LOM 20
                    LOM 21
20   LOM 22
                    LOM 23
                    LOM 24
                    LOM 25
                    LOM 26
25   LOM 27
                    LOM 28
                    LOM 29

The drawings are presented to show the location of the
30   DEPs by reference to Figure 1 and the differential
expression in Figures 2-8.

The locations are shown in the maps of Figures 1A - 1E,

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in which Figure 1E shows the entire map from the 2-D-PAGE
of liver tissue of lean mice. (It so happens that all the
differentially expressed proteins identified are over-
expressed in the lean control mice, relative to the ob/ob

5   control mice.) The map is divided into four quadrants in
the manner:

Fig. 1A	Fig. 1B
Fig. 1C	Fig. 1D

10   in which the pI runs from left (low pI) to right (high
pI) and the relative molecular mass from top to bottom,
so that the quadrant of Figure 1A shows the spots
corresponding to proteins of the lowest pI and highest
relative molecular mass and the quadrant of Figure 1D

15   those of the highest pI and lowest relative molecular
mass.   The DEPs are marked as LOM16, LOM17, LOM18,
LOM19, LOM20, LOM 21, LOM22, LOM23, LOM24, LOM25, LOM26,
LOM27, LOM28, and LOM29. The other spots identified are
for reference purposes and have numbers of five digits

20   preceded by "P" or "Q", corresponding to the SWISS-PROT
accession number, thus linking the spots to their genes.
The following Tables 1 and 2 list the characteristics of
the DEP spots in terms of relative volumes, areas, and
optical densities and apparent pI and relative molecular

25   mass.

Table 1: DEP spots from liver of obese mice

	ID	% Vol.	Area	OD	Pi	RMM
	LOM16	0.177	1.470	1.460	6.35	160851
	LOM17	0.034	0.888	0.595	5.61	90000
5	LOM18	0.059	0.858	0.959	6.54	82896
	LOM19	0.370	3.154	1.414	5.31	58000
	LOM20	0.295	2.603	1.438	5.83	56422
	LOM21	0.409	3.307	1.475	6.31	49972
	LOM22	0.283	2.328	1.492	6.25	42937
10	LOM23	0.245	1.960	1.534	6.80	36508
	LOM24	0.293	2.419	1.462	6.83	32671
	LOM25	0.388	3.277	1.439	6.26	26266
	LOM26	0.298	2.664	1.398	5.10	24202
	LOM27	0.168	2.174	1.012	7.17	15283
15	LOM28	1.495	10.596	1.655	9.07	13016
	LOM29	0.255	2.756	1.200	6.33	11946

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Table 2: Reference spots from liver of obese control
mice

	ID	% Vol.	Area	OD	Pi	RMM
	P99015	0.122	1.102	1.286	6.31	160851
5	P99015	0.177	1.470	1.460	6.35	160851
	P99015	0.161	0.766	1.076	6.17	160851
	P99015	0.098	1.041	1.252	6.22	160851
	P99015	0.157	1.715	1.302	6.26	160000
	Q05920	0.085	1.133	1.050	6.16	121447
10	Q05920	0.039	0.643	0.803	6.12	121447
	Q05920	0.201	2.266	1.281	6.21	120000
	POSI13	0.023	0.306	0.981	4.87	90604
	POSI13	0.082	0.827	1.214	4.89	89679
	P08113	0.025	0.337	0.911	4.89	90000
15	P99017	0.011	0.766	0.176	5.42	90604
	P99017	0.020	1.194	0.228	5.52	90000
	P99017	0.034	0.088	0.595	5.61	90000
	P99017	0.094	1.317	0.077	5.68	89359
	P99017	0.020	0.898	0.298	5.77	90000
20	P11499	0.033	0.766	0.669	5.02	82305
	P37040	0.020	0.674	0.465	5.28	71490
	P37040	0.008	0.429	0.226	5.25	71299
	P20029	0.030	0.735	0.553	4.99	70356
	P20029	0.074	0.919	1.072	5.01	70169
25	P20029	0.433	3.951	1.391	5.04	69426
	P38647	0.044	1.102	0.539	5.29	69426
	P38647	0.011	0.643	0.200	5.27	69426
	P38647	0.067	1.133	0.861	5.32	69057
	P38647	0.266	2.909	1.273	5.34	68690
30	P16627	0.027	1.164	0.317	5.25	67781
	P16627	0.066	0.949	0.970	5.27	67421
	P16627	0.367	3.675	1.304	5.30	67063
	P07724	0.022	0.888	0.343	5.68	66707

| P07724 | 0.133 | 1.378 | 1.256 | 5.79 | 66529 |
| P07724 | 0.036 | 1.072 | 0.469 | 5.42 | 66000 |

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ID	% Vol.	Area	OD	Pi	RMM
P07724	0.206	1.929	1.380	5.49	65673
P14733	0.020	0.766	0.366	5.20	65510
P14211	0.173	2.113	1.107	4.44	59607
P19226	0.050	1.072	0.614	5.26	58289
P19226	0.370	3.154	1.414	5.31	58000
P19226	0.132	1.501	1.181	5.29	58144
P14211	0.023	0.949	0.346	4.43	56578
P27773	0.077	1.317	0.893	5.70	56422
P27773	0.295	2.603	1.438	5.83	56422
P09103	0.422	3.246	1.471	4.86	55496
P09103	0.059	0.551	1.332	4.82	55803
P09103	0.114	1.001	1.421	4.88	55496
P99019	0.096	1.072	1.252	5.72	54585
P02551	0.050	0.827	0.786	5.11	53541
P04104	0.043	1.041	0.556	5.54	53246
P20152	0.026	0.521	0.574	5.12	53099
P56480	0.054	1.072	0.654	4.96	49835
P56480	0.137	1.409	1.316	5.00	49561
P56480	0.448	3.553	1.472	5.05	49152
P17182	0.468	3.706	1.542	6.34	46771
P05784	0.038	1.011	0.497	5.15	46771
P05784	0.083	1.501	0.737	5.20	46771
P05784	0.130	1.654	1.056	5.26	46642
P29758	0.074	1.072	1.017	5.88	44260
P99021	0.318	2.695	1.392	5.25	42000
P99022	0.064	0.827	1.093	5.27	42232
P99016	0.160	1.715	1.218	6.43	41168
P35505	0.416	3.093	1.635	6.77	40985
P14206	0.111	1.562	0.991	4.85	40985
P99018	0.016	1.164	0.175	5.27	40622
Q64374	0.484	4.104	1.367	5.08	33929
P50431	0.058	0.521	1.316	6.78	32060

| P16015 | 1.493 | 9.769 | 1.722 | 6.93 | 28149 |
| P16015 | 0.560 | 4.165 | 1.576 | 6.84 | 27855 |

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ID	% Vol.	Area	OD	Pi	RMM
P16015	0.329	2.909	1.406	6.74	27914
P00405	0.071	1.409	0.699	4.72	26711
Q00623	0.020	1.072	0.243	5.45	24664
Q00623	0.054	1.592	0.475	5.27	24202
P14701	0.077	1.746	0.588	4.81	24050
P02762	0.173	1.776	1.197	4.80	19046
P02762	0.305	2.542	1.461	4.88	19046
P99014	0.044	3.553	0.152	4.08	14659
P56395	0.236	3.032	1.069	4.83	14567
P08228	0.468	4.349	1.316	6.14	14537
P12787	0.224	2.848	1.046	4.91	12433
P16045	0.173	2.634	0.915	5.06	12381
P12710	0.255	2.756	1.200	6.33	11946

The positions of some of the DEPs coincide with those of
reference spots. Sequencing will be undertaken to check
whether they are actually the same.

Referring now to Figures 2-8, these Figures show images
of lean controls (left) and ob/ob ("obese") controls
(right) relating to one mouse. It should be appreciated
that these images cannot fully represent to the eye the
differences in expression measurable by computer. Thus,
underneath, a bar chart is provided in which the volume
of the spot as a percentage of the total volume of all
spots is shown on the y-axis. All bar charts relate to
4 mice and resulted from a student T test ($P<0.01$).

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The following proteins have been identified as liver markers.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
LOM16	CPS1	Carbamoyl-phosphate synthetase I	P07756/P99015	PMF+MS/MS
LOM17	FTHFD	10-Formyltetrahydrofolate dehydrogenase	P28037/P99017	PMF
LOM18	M2GD	Dimethylglycine dehydrogenase	Q63342	PMF
LOM19	HSP60	Heat shock protein 60	P19226	PMF
LOM20	ERP60	Protein disulfide isomerase ER-60	P27773	PMF
LOM21	ALDH1	Aldehyde dehydrogenase, mitochondrial	P47738	PMF
LOM22	SAHH	Adensoylhomocysteinase	P50247	PMF
LOM23	ALR	Alcohol dehydrogenase	P51635	PMF+MS/MS
LOM24	GNMT	Glycine N-methyltransferase	P13255	PMF+MS/MS
LOM25	AOP2	Nonselenium glutathione peroxidase	O08709	PMF+MS/MS
LOM26	-	Lactoyl glutathione lyase	-	-
LOM27	PPIA	Peptidyl-prolyl cis-trans isomerase A	P17742	PMF+MS/MS
LOM28	FABPL	Fatty acid binding protein, liver	P12710	PMF+MS/MS
LOM29	DDT	D-dopachrome tautomerase	O35215	PMF+MS/MS
LSEM30	-	Unknown protein	P99032	PMF

Preparative 2-D-PAGE

The analytical 2-DGE described above was repeated, with the following changes. Four mg of dried liver was mixed with 450 microlitres of the solubilisation solution and loaded into the IPG strips by in-gel rehydration.

After the first dimension run the strips were equilibrated using 3 ml of each buffer per groove.

Protein electroblotting

The blotting of proteins separated by 2-D-PAGE onto

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polyvinylidene difluoride (PVDF) membranes has enabled the
identification and characterisation of proteins from complex
biological samples. Transfer of the proteins can be carried
out using several methods such as vacuum, capillary or
5   electric field. Electroblotting, using vertical buffer tanks
or a semi-dry method is preferred. Both techniques can use
the 3-[cyclohexamino]-1-propanesulfonic acid (CAPS) transfer
buffer. Gloves must be worn and all filter papers should be
washed three times for 3 min in water and three times in
10   transfer buffer. These two steps are important in order to
avoid any protein or amino acid contamination.

The procedure was as follows. After second-dimensional
electrophoresis, the gels were soaked in deionized water for
15   3 min. Then they were equilibrated in a solution containing
10mM CAPS pH 11 for 30 min. At the same time, PVDF membranes
were wetted in methanol for 1 min and equilibrated in a
solution containing 10mM CAPS pH 11 and methanol (10% v/v)
also for 30 min. Electroblotting was carried out in a semi-
20   dry apparatus with a solution containing 10mM CAPS pH 11 and
methanol (20% v/v anodic side; 5% v/v cathodic side) at 1
mA/cm^2 constant current for 3 hours at 15°C.

Protein detection on PVDF membranes

25   Amido Black and Coomassie Brilliant Blue R-250 were used
instead of silver staining to visualise proteins on PVDF
membranes and are compatible with the ensuing post-separation
analysis. Thus, in another 2-DGE run, after electrotransfer,
the PVDF membranes were stained in a solution containing
30   Amido Black (0.5% w/v), isopropanol (25% v/v) and acetic acid
(10% v/v) for 2 min. Destaining was done by several soakings
in deionized water.

In another run, after electrotransfer, the PVDF membranes
were stained in a solution containing Coomassie Brilliant
Blue R-250 (0.1% w/v) and methanol (50% v/v) for 15 min.
Destaining was done in a solution containing methanol (40%
5   v/v) and acetic acid (10% v/v). The same method was used for
preparative gels that did not need electrotransfer for
further post-separation analysis, such as peptide mass
fingerprinting.

10   The PVDF stained membranes were either air-dried or dried on
a 3mm thick plate onto a heating plate at 37°C for 10 min.

Scanning
This was done as described above.
15

Protein identification
In amino sequence analysis by Edman degradation, amino acid
derivatives are sequentially cleaved one at a time from the
protein. Proteins with a chemically inaccessible alpha-amino
20   group cannot be sequenced directly by this procedure and are
termed N-terminally blocked. The best way to overcome the
blocked proteins is to generate individual fragments by
chemical or proteolytic cleavage. Routinely, ten to twelve
Edman degradation cycles were performed for each spot. A
25   search in the SWISS-PROT database was made to detect identity
to known protein sequences.

The Amido Black stained proteins were excised with a razor
blade and N-terminal sequencing was performed using an ABI
30   model 473A or 477A microsequencer from Applied Biosystems
equipped with "'Problott" cartridges.

For internal sequencing, the spots of interest were excised

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and soaked for two hours in a solution containing acetic acid
(100mM), methanol (10% v/v) and PVP-40 (1% v/v) at 37°C. After
three washes in deionized water, the PVDF spots were cut into
small pieces (about 1mm²) and incubated in 25 microlitres of a
5   solution containing sodium phosphate (100mM) pH 8.0 and lysyl
endopeptidase (1 microgram). Following overnight digestion
at room temperature, guanidine-HCl (28 mg) and DTT (100
micrograms) were added. After reduction for 2 hours at 37°C,
the mixture was incubated for 30 min, at room temperature,
10   with 300 micrograms of iodoacetamide. The digestion solution
was removed and kept. PVDF pieces were then extracted
overnight with 25 microlitres of a solution containing
isopropanol (70% v/v) and trifluoroacetic acid (5% w/v).
This elution solution was removed and the PVDF was washed
15   twice with 60 microlitres of TFA (0.1% w/v). The digestion
and elution solutions were pooled together with two final
washes and this mixture was separated by two-dimensional
reverse phase HPLC and sequence determination performed.

20   **Immunoblotting**

PVDF membranes were first stained to visualise proteins,
following which the immunodetection was undertaken. This
allowed matching of proteins detected with ECL against those
detected with the non-specific protein stain through computer
25   comparison of both images. The mechanical strength of PVDF
was also exploited as the same 2-D gel can be used many times
for different antibodies.

The whole procedure was carried out in a rotating oven at
30   room temperature. The use of a nucleic acid glass hybridiser
tube minimised the volumes and costs.

•   The membranes were blocked in 10 ml of a solution of 30

PBS (pH 7.2) and non-fat dry milk (5% w/v) for 30 min.

- The membranes were then incubated in 10 ml of a solution containing PBS-"'Tween" 20 (0.5% v/v), non-fat dry milk (5% w/v) and the primary antibody/antibodies (1:100 or greater, depending on the antibody) for 2 hours.

- Three quick rinses were performed with 10 ml of PBS-"Tween" 20 (0.5% v/v) and then the membranes were washed for 3 x 10 min with 10 ml of PBS-"Tween" 20 (0.5% v/v).

- The membranes were incubated in 10 ml of a solution containing PBS-"Tween" 20 (0.5% v/v), non-fat dry milk (5% w/v) and the secondary peroxidase-conjugated antibody (1.1000; for example, if the primary antibody was sheep anti-mouse, then goat anti-sheep IgG was used as the secondary antibody) for 1 hour.

- Three quick rinses were performed with 10 ml of PBS-"Tween" 20 (0.5% v/v) and then the membranes were washed for 5 x 10 min with 10 ml of PBS-"Tween" 20 (0.5% v/v).

- After the last wash, the membranes were transferred to a clean glass plate and covered with 10 ml of developing solution (for example ECL from Amersham International or Roche Diagnostics) for 2 min.

- The excess developing solution was drained, the 20 membranes were wrapped in "Saran" film and fixed in an X-ray film cassette with the proteins facing up.

- An X-ray film was then exposed in a dark room for few seconds or up to several minutes.

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Peptide mass fingerprinting

The 2-DGE method was repeated, but using a Coomassie blue
stain. The 2-DGE spots were destained with 100 microlitres
of 30% acetonitrile in 50mM ammonium bicarbonate at 37°C for
45 min. The supernatant was discarded and the gel spots
dried in a "SpeedVac" for 30 min. The gel spots were
rehydrated with 25 microlitres of a solution containing 0.2
micrograms of porcine trypsin and 50mM ammonium bicarbonate
for 2 hours at 35°C. Then the gel spots and supernatant were
dried in a "SpeedVac" for 30 min, rehydrated with 20
microlitres of H_2O for 30 min at 35°C and dried again for 30
min. Twenty microlitres of a solution of 50% of acetonitrile
and 0.1% of TFA was added to the spots and sonicated for 10
min. Two microlitres of the supernatants was loaded in each
well of a 96 or 400 MALDI target plate. The samples were
air-dried. Then 2 microlitres of a solution containing 4
mg/ml of alpha-cyano-4-hydroxycinnamic acid, 50% acetonitrile
and 0.1% TFA was added to each well and air-dried.

The peptide mixtures were analysed by matrix-assisted laser
desorption/ionization time-of-flight mass spectrometer
(Perseptive Biosystems Voyager Elite MALDI-TOF-MS) with a
nitrogen laser (337nm) and operated in reflectron delayed
extraction mode.

Protein identification has been carried out using "PeptIdent"
(**http://www.expasy.ch/tools/peptident.html**). It is a tool that
allows the identification of proteins using pI, relative
molecular mass and peptide mass fingerprinting data.
Experimentally measured, user-specified peptide masses were
compared with the theoretical peptides calculated for all
proteins in the SWISS-PROT/TREMBL databases.

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MS/MS sequencing

When protein identification was not successful with the
peptide mass fingerprinting procedure, the supernatant of
5   digested spots was desalted in "ZipTip" C18 pipette tips
(Millipore) and eluted with 50% acetonitrile and 0.1% TFA.
Peptides were applied by nanoflow (in-house nanospray) sample
introduction to a tandem mass spectrometer that consists of
two quadrupoles and an orthogonal time of flight tube (Q-TOF)
10   from MicroMass (UK). Fragment ion spectra were interpreted
with the MOWSE database search
(http://www.seqnet.dl.ac.uk/mowse.html).

Data management: The mouse SWISS-2-DPAGE database

15   SWISS-2-DPAGE is an annotated 2-D-PAGE database in which all
the data are easily retrieved by computer programs and stored
in a format similar to that of the SWISS-PROT Protein
Sequence Database, one of the most updated and annotated
protein sequence databases presently available. The SWISS-2-
20   DPAGE database assembles data on proteins identified on
various 2-D-PAGE maps. Each SWISS-2-DPAGE entry contains
data on one protein, including mapping procedures,
physiological and pathological data and bibliographical
references, as well as several 2-D-PAGE images showing the
25   protein location. Cross-references are provided to SWISS-
PROT and, through the latter, to other databases (EMBL,
Genbank, PROSITE, OMIM, etc). The database has been set up
on the ExPASy World Wide Web server (http://www.expasy.ch/).
Worldwide, scientists using similar 2-D-PAGE protocols
30   (immobilised pH gradient as first dimensional separation) are
now able to compare their images with SWISS-2-DPAGE maps.

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EXAMPLE 2

The method of Example 1 was repeated, substituting for the
liver tissue 200 micrograms (analytical scale) or 4 mg
(preparative scale) of dried gastrocnemius muscle tissue and
5   using the same respective volumes of the protein-solubilising
solution as in Example 1. A 2-DGE map was thus obtained,
from which DEPs were identified and isolated.

The muscle DEPs were:
10   MOM 31
MOM 32
MOM 34
MOM 36
which were underexpressed in ob/ob mouse skeletal muscle
15   relative to expression in lean mouse skeletal muscle and
MOM 33
MOM 35
which were overexpressed in ob/ob skeletal muscle relative to
lean. These are shown in Figures 9-11 inclusive.
20

The location of the DEPs has been determined by running the
liver extract from lean mice in the same gel as the muscle
extract, so that liver protein reference spots appear in the
muscle extract gel to serve as reference spots.
25

Table 3 gives the characteristics of the DEP spots in terms
of relative volumes, areas, optical densities and apparent pI
and relative molecular mass.

30

Table 3

Markers	Vol.	% Vol.	Area	% OD	OD	pI	Mw
MOM31	0.259	0.075	0.919	1.225	1.515	6.21	118405
MOM32	0.339	0.098	1.347	1.087	1.344	6.25	101518
MOM33	0.072	0.021	0.643	0.459	0.567	5.19	89359
MOMT35	0.095	0.028	1.011	0.43	0.532	5	40803
MOM34	0.231	0.067	1.133	0.954	1.18	6.19	38941
MOM36	0.091	0.026	1.378	0.301	0.372	6.17	32127

The following protein have now been identified as muscle markers.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
MOM31	PCX	Pyruvate carboxylase	Q05920	PMF
MOM32	-	2-oxoglutarate dehydrogenase E1 component	-	-
MOM33	-	-	-	-
MOM34	IRP2	Iron responsive element binding protein 2	Q62751	PMF
MOM35	-	-	-	-
MOM36	2	-	-	-

EXAMPLE 3

The method of Example 1 was repeated, substituting for the liver tissue 16mg (analytical scale) or 160mg (preparative scale) of white adipose tissue and using the same respective volumes of the protein-solubilising solution as in Example 1. A 2-DGE map was thus obtained, from which DEPs were identified and isolated.

The location of the proteins has been determined by running the liver extract from lean mice in the same gel as the white

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adipose extract, so that the liver protein reference spots
appear in the white adipose extract get to serve as reference
spots for the latter also.

5   The following differentially expressed proteins are
underexpressed in ob/ob mouse adipose tissue relative to lean
mouse adipose tissue:

WOM 37	WOM 52	WOM 60
WOM 38	WOM 53	WOM 61
WOM 44	WOM 54	WOM 62
WOM 45	WOM 55	WOM 63
WOM 48	WOM 57	WOM 64
WOM 49	WOM 58	
WOM 50	WOM 59	

10

15

The following differentially expressed proteins were
overexpressed in obese mouse adipose tissue relative to
adipose tissue of lean mice:

WOM 39
20   WOM 40
WOM 41
WOM 42
WOM 43
WOM 46
25   WOM 47
WOM 51

These are shown in Figures 12-23 inclusive

The characteristics of the DEP spots in terms of relative
30   volumes, areas, and optical densities and apparent pI and
relative molecular mass are given in Table 4.

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Table 4

Markers	Vol.	% Vol.	Area	% OD	OD	pI	Mw
WOMT37	0.527	0.088	1.164	0.817	2.292	6.11	122912
WOM38	0.724	0.12	1.654	0.764	2.145	6.97	81427
WOMT39	0.161	0.027	0.643	0.563	1.581	6.28	68965
WOM40	0.744	0.124	1.684	0.83	2.328	5.34	67063
WOMT42	0.277	0.046	0.827	0.576	1.615	5.35	62580
WOM41	0.174	0.029	0.735	0.493	1.383	5.29	60937
WOM43	0.354	0.059	0.766	0.829	2.326	5.12	53967
WOM46	0.141	0.024	1.011	0.234	0.657	5.15	37256
WOMT48	0.18	0.03	0.827	0.424	1.191	6.16	36889
WOM47	0.329	0.055	1.96	0.3	0.841	5.19	36526
WOMT49	0.364	0.061	0.949	0.749	2.101	6.37	35371
WOMT50	9.187	1.529	13.934	1.094	3.069	6.97	32129
WOM51	0.637	0.106	1.439	0.865	2.426	5.35	31989
WOM52	1.644	0.274	2.695	1.037	2.909	6.5	26760
WOM55	0.235	0.039	1.072	0.471	1.322	6.29	22380
WSEM65	0.128	0.021	1.746	0.124	0.349	5.39	22172
WOM54	0.195	0.032	1.409	0.269	0.755	5.66	22275
WOM53	0.446	0.074	1.47	0.619	1.736	6.01	22069
WOM56	0.025	0.004	0.613	0.066	0.184	5.53	19279
WOM57	0.177	0.029	1.133	0.311	0.873	6.5	16454
WOM59	0.703	0.117	1.654	0.803	2.252	7.75	14353
WOM60	1.17	0.195	2.328	0.908	2.548	6.6	13775
WOM61	0.607	0.101	1.347	0.814	2.283	7.51	13695
WOM58	0.068	0.011	1.133	0.109	0.305	5.42	13416
WOM62	0.621	0.103	1.868	0.673	1.887	6.28	11151
WOM64	0.484	0.081	1.531	0.556	1.561	6.6	10515
WOM63	0.571	0.095	1.501	0.647	1.816	6.68	10546

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The following adipose markers have been identified.

MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
WOMT37				
WOM38				
WOMT39				
WOM40	GRP75	Mitochondrial stress-70 protein	P38647	PMF + MS/MS
WOM41				
WOMT42	ALB	Albumin (Fragment)	P07724	PMF + MS/MS
WOM43				
WOM44				
WOM45				
WOM46				
WOM47				
WOMT48				
WOMT49				
WOMT50	CA3	Carbonic anhydrase 3 (fragment)	P16015	PMF + MS/MS
WOM51	ANX2	Lipocortin II	P07356	PMF + MS/MS
WOM52	CA3	Carbonic Anhydrase 3 (Fragment)	P16015	PMF
WOM53				
WOM54				
WOM55				
WOM56	FTL1	Ferritin Light Chain 1	P29391	PMF + MS/MS
WOM57				
WOM58				
WOM59				
WOM60				
WOM61	PPcA	Peptidyl-prolyl cis-trans isomerase A	P17742	MS/MS
WOM62	GST P2	Glutathione-S transferase P1 (fragment)	P19157	MS/MS
WOM63				
WOM64	CA2	Carbonic Anhydrase 2 (Fragment)	P00920	PMF + MS/MS
WSEM65				

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EXAMPLE 4

The method of Example 1 was repeated, substituting for the
liver tissue 400 micrograms (analytical scale) or 4mg
(preparative scale) of brown adipose tissue and using the

5   same respective volumes of the protein-solubilising solution
as in Example 1. The weight of sample loaded was 150
micrograms (analytical) or 1.5mg (preparative). A 2-DGE map
was thus obtained, from which DEPs were identified and
isolated.

10

The location of the proteins has been determined by running
the liver extract from lean mice in the same gel as the brown
adipose extract, so that the liver protein reference spots
appear in the brown adipose extract gel to serve as reference

15   spots for the latter also.

The brown adipose tissue DEPs which are overexpressed in
ob/ob mouse brown adipose tissue relative to lean mouse brown
adipose tissue are:

20   BOM 66
             BOM 67
             BOM 68
             BOM 75
             BOM 76

25   BOM 77

The brown adipose tissue DEPs that are underexpressed in
ob/ob mouse brown adipose tissue relative to lean mouse
adipose tissue are:

30   BOM 69
             BOM 70
             BOM 71
             BOM 72

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BOM 73

BOM 74

The characteristics of the DEP spots in terms of relative
5   volumes, areas, and optical densities and apparent pI and
relative molecular mass are given in Table 5.

Table 5

Markers	Vol.	% Vol	Area	% OD	OD	pI	Mw
BOM66	0.592	0.161	2.082	1.178	1.455	6.44	73811
BOM67	0.043	0.012	0.643	0.329	0.407	5.78	54647
BOMT68	0.047	0.013	1.194	0.18	0.223	5.68	54476
BOM69	0.485	0.132	1.623	1.369	1.691	5.13	49744
BOM71	1.125	0.306	3.277	1.622	2.004	6.64	44857
BOM70	0.635	0.173	2.205	1.246	1.539	6.4	44857
BOM72	0.483	0.131	1.868	1.061	1.311	6.8	38472
BOM73	0.533	0.145	1.654	1.479	1.827	5.18	24316
BOM74	0.048	0.013	1.654	0.123	0.152	4.97	12722
BOM75	0.009	0.002	0.398	0.271	0.335	5.88	12432
BOMT76	6.325	1.722	10.627	2.369	2.927	7.61	12093
BOM77	0.55	0.15	2.787	0.861	1.064	5.14	12111

(10, 15, 20 are line numbers in the left margin)

These are shown in Figures 24-29 inclusive.

25   The following brown adipose tissue markers have been
identified:

30

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MARKER	GENE NAME	PROTEIN DESCRIPTION	SWISS-PROT AC	METHOD OF IDENTIFICATION
BOM66	ACO2	Aconitate Hydratase (human)	Q99798	MS/MS
BOM67	-	-	-	-
BOMT68	-	-	-	-
BOM69	-	-	-	-
BOM70	-	-	-	-
BOM71	ACO2	Aconitate Hydratase Fragment (human)	A99798	MS/MS
BOM72	FH	Fumarate Hydratase (dimer)	P97807	MS/MS
BOM73	-	-	-	-
BOM74	-	-	-	-
BOM75	-	-	-	-
BOMT76	-	-	-	-
BOMT76	GBP	Lactose-binding Lectin 1	P16045	MS/MS

EXAMPLE 5

Lean mice and ob/ob mice, either fed ad-lib or fasted overnight were anaesthetised with 50% hypnovel and 50% hyponorm and then killed humanely with carbon dioxide gas. The hypothalamus was removed and frozen in liquid nitrogen. The method of example 1 was then followed but using 200mg of hypothalamic tissue rather than 200mg liver.

The references mentioned herein are all expressly incorporated by reference.

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Claims:

1.   A method of screening an agent to determine its
usefulness in treating a condition characterised by abnormal
body weight or eating dysfunction, the method comprising:

5   (a)   establishing a paradigm in which at least one protein
is differentially expressed in relevant tissue from, or
representative of, subjects having differential levels of
body weight or eating dysfunction;

           (b)   obtaining a sample of relevant tissue taken from, or
10   representative of, a subject having body weight or eating
disorders, who or which has been treated with the agent being
screened;

           (c)   determining the presence, absence or degree or
expression of the differentially expressed protein or
15   proteins in the tissue from, or representative of, the
treated subjects; and

           (d)   selecting or rejecting the agent according to the
extent to which it changes the expression of the
differentially expressed protein or proteins in the treated
20   subject having body weight or eating disorders.

2.   The method of claim 1, wherein the agent is selected if
it converts the expression of the differentially expressed
protein towards that of a subject having a more normal body
25   weight or eating behaviour.

3.   The method of claim 1 or claim 2, wherein the agent is
selected if it converts the expression of the protein or
proteins to that of the normal subject.

30

4.   The method of any one of claims 1 to 3, wherein the body
weight or eating disorder is a result of a disorder which
causes an increase in body weight and/or which is associated

with an excess food consumption.

5.   The method of any one of the preceding claims, wherein
the paradigm is based on tissue from obese subjects and
normal subjects.

6.   The method of any one of the preceding claims, wherein
the paradigm is based on a comparison of subcutaneous and
omental adipose tissue from the same individuals.

7.   A method of any of claims 1 to 4, wherein the body
weight and/or eating disorder is a result of a disorder which
causes a reduction in body weight and/or which is associated
with a low food intake.

8.   A method of claim 7, wherein the paradigm is based on
tissue from subjects with anorexia nervosa or bulimia or AIDS
or cancer and normal subjects.

9.   The method of claim 1, whereon the paradigm is based on
animals which are models of obesity as a result of a genetic
mutation such as ob/ob, db/db, agouti, fat, tub, fa/fa
together with lean littermates.

10.   The method of claim 1, wherein the paradigm is based on
animals in which obesity is induced or exacerbated by dietary
treatment.

11.   The method of claim 1, wherein the paradigm is based on
lean and obese animals obtained by a selective breeding
programme froma common stock.

12.   The method of claim 1, wherein the paradigm is based on

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desert rodents such as spiny mice or sand rats, which develop
obesity on normal laboratory diets.

13. The method of claim 1, wherein differential levels of
obesity occur in apparently similar animals in which it is
attempted to induce obesity by dietary modification.

14. The method of any one of the preceding claims, wherein
in the paradigm, the subjects having differential levels of
body weight or function comprise normal subjects and obese
subjects.

15. A method of any one of the preceding claims, wherein in
the paradigm, the subjects having differential levels of body
weight comprise obese subjects and body weight reduced
previously obese subjects or obese subjects and subjects who
resisted the development of obesity.

16. The method of any one of the preceding claims, wherein
in the paradigm, the subjects having differential levels of
body weight comprise normal subjects and subjects having a
below normal body weight.

17. The method of claim 15, wherein the body weight of a
subject is reduced by treatment with a drug, dietary
restriction or exercise.

18. The method of claim 17, wherein the drug is a
thermogenic drug.

19. The method of claim 18, wherein the thermogenic drug is
a b_3-adrenoceptor agonist or leptin.

20. The method of claim 17, wherein the drug is an anorexic drug.

21. The method of claim 20, wherein the anorexic drug is sibutramine or fenfluramine or leptin.

22. The method of claim 1 to 4, wherein the paradigm is based on animals, which are fed, fasted or sated.

23. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise normal subjects and underweight or overweight subjects.

24. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise:

    (a) normal subjects and underweight or overweight subjects; and

    (b) underweight or overweight subjects which have not been treated with the agent and underweight or overweight subjects which have been treated with the agent.

25. The method of claim 24, wherein the differential levels of protein expression are not observed in normal subjects who have and have not been treated with the agent.

26. The method of any one of the preceding claims, wherein in the paradigm, the subjects having differential levels of protein expression comprise:

    (a) normal subjects who have and have not been treated with the agent; and

    (b) subjects having body weight and/or eating disordered

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function who have and have not been treated with the agent.

27.   The method of claim 26, wherein the differential levels
of protein expression are not observed in normal subjects and
subjects having body weight and/or eating disordered
function, both groups of subject being untreated with the
agent.

28.   The method of any one of the preceding claims, wherein
the paradigm is established using two-dimensional gel
electrophoresis carried out on the relevant tissue or a
protein-containing extract thereof.

29.   The method of any one of the preceding claims, further
comprising the step of isolating a differentially expressed
protein identified in the method.

30.   The method of claim 29, further comprising the step of
characterising the isolated protein.

31.   The method of any one of the preceding claims, wherein
the differentially expressed protein or proteins comprise one
or more of MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35,
WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM
54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64,
WOM 49, WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM
42, WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68,
BOM 75, BOM 76, BOM 77, BOM 69, BOM 70, BOM 71, BOM 72, BOM
73, BOM 74.

32.   The method of claim 30 or 31, further comprising using
the protein in an assay for specific binding partners of the
protein.

33. The method of claim 30 or claim 31, further comprising using the protein in an assay to screen for agonists or antagonists of the protein.

34. The method of any one of claims 1 to 33, wherein the agents or proteins are screened using a high throughput screening method.

35. A method of making a pharmaceutical composition which comprises having identified an agent using the method of any one of claims 1 to 28, the further step of manufacturing the agent and formulating it with an acceptable carrier to provide the pharmaceutical composition.

36. A protein for use in a method of medical treatment wherein the protein is selected from MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM 49, WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM 42, WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68, BOM 75, BOM 76, BOM 77, BOM 69, BOM 70, BOM 71, BOM 72, BOM 73, BOM 74.

37. Use of an agent identified by the method of any one of claims 1 to 28 for the preparation of a medicament for the treatment of a condition characterised by body weight and/or eating dysfunction.

38. The use of claim 37 wherein the condition is obesity, non-insulin dependent diabetes, anorexia nervosa, bulimia or cachexia induced by AIDS or cancer or trauma.

39. The use of claim 37 or claim 38, wherein the

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differentially expressed protein or proteins comprise one or
more of MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM
37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54,
WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM
5   49, WOM 58, WOM 50, WOM 59,   WOM 39, WOM 40, WOM 41, WOM 42,
WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68, BOM
75, BOM 76, BOM 77, BOM 69, BOM 70, BOM 71, BOM 72, BOM 73,
BOM 74.

10   40. A method of treating a condition characterised by body
weight and/or eating dysfunction in a patient, the method
comprising administering a therapeutically or
prophylactically effective amount of such an agent identified
by a method of any one of claim 1 to 28 to the patient.
15

41. The method of claim 40, wherein the body weight and/or
eating dysfunction is a result of obesity, non-insulin
dependent diabetes or type 2 diabetes, anorexia nervosa,
bulimia or cachexia induced by AIDS or cancer or trauma.
20

42. A method of determining the nature or degree of body
weight and/or eating dysfunction in a human or animal
subject, the method comprising:
    (a) establishing a paradigm in which at least one protein
25   is differentially expressed in relevant tissue from, or
representative of, subjects having differential levels of
body weight and/or eating function;
    (b) obtaining a sample of the tissue from the subject;
    (c) determining the presence, absence or degree of
30   expression of the differentially expressed protein or
proteins in the sample; and
    (d) relating the determination to the nature or degree of
the body weight or eating dysfunction by reference to a

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previous correlation between such a determination and
clinical information.

43. The method of claim 42, wherein the sample is a tissue
sample or body fluid sample or urine.

44. The method of claim 42 or claim 43, wherein in the
paradigm at least four proteins are differentially expressed,
providing a multi-protein fingerprint of the nature or degree
of the body weight or eating dysfunction.

45. The method of any one of claims 42 to 44, which further
comprises determining an effective therapy for treating the
body weight or eating dysfunction.

46. A method of treatment by the use of an agent that will
restore the expression of one or more differentially
expressed proteins in the body weight or eating dysfunction
state to that found in the normal state in order to prevent
the redevelopment of obesity in body weight reduced
previously obese subjects.

47. A method whereby the pattern of differentially expressed
proteins in a tissue sample or body fluid sample or urine of
an individual with body weight eating dysfunction is used to
predict the most appropriate and effective therapy to
alleviate the body weight or eating dysfunction state and to
monitor the success of that treatment.

48. A method of claim 47, wherein the body weight or eating
dysfunction state is obesity.

49. A protein which is differentially expressed in relevant

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tissue from, or representative of subjects having
differential levels of body weight or eating dysfunction and
which is as obtainable by the method of two-dimensional gel
electrophoresis carried out on said tissue or a protein-
5   containing extract thereof, the method comprising:

(a)   providing non-linear immobilized pH gradient (IPG)
strips of acrylamide polymer 3 mm x 180 mm;

(b)   rehydrating the IPG strips in a cassette containing 25
ml. of an aqueous solution of urea (8M), 3-
10   [(cholamidopropyl)dimethylammonio]-1-propanesulphonate
(CHAPS, 2% w/v), dithioerythritol (DTE, 10mM), mixture of
acids and bases of pH 3.5 to 10 (2% w/v) and a trace of
Bromophenol Blue;

(c)   emptying the cassette of liquid, transferring the
15   strips to an electrophoretic tray fitted with humid electrode
wicks, electrodes and sample cups, covering the strips and
cups with low viscosity paraffin oil;

(d)   applying 200 micrograms of an aqueous solution of
dried, powdered material of the relevant body tissue in urea
20   (8M), CHAPS (4% w/v), Tris (40 mM), DTE (65 mM), SDS (0.05%
w/v) and a trace of Bromophenol Blue to the sample cups, at
the cathodic end of the IPG strips;

(e)   carrying out isoelectric focusing on the gel at a
voltage which increases linearly from 300 to 3500 V during 3
25   hours, followed by another 3 hours at 3500 V, and thereafter
at 5000V for a time effective to enable the proteins to
migrate in the strips to their pI-dependent final positions;

(f)   equilibrating the strips within the tray with 100 ml
of an aqueous solution containing Tris-HCl (50 mM) pH 6.8,
30   urea (6M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v)
for 12 minutes;

(g)   replacing this solution by 100 ml. of an aqueous
solution containing Tris-HCl (50 mM) pH 6.8, urea (6M),

glycerol (30% v/v), SDS (2% w/v), iodoacetamide(2.5% w/v) and a trace of Bromophenol Blue for 5 minutes;

(h)   providing a vertical gradient slab gel 160 x 200 x 1.5 mm of acrylamide/piperazine-diacrylyl cross-linker(9-16%T/2.6%C), polymerised in the presence of TEMED (0.5% w/v), ammonium persulphate (0.1% w/v) and sodium thiosulphate (5 mM), in Tris-HCl (0.375M) pH 8.8 as leading buffer;

(i)   over-layering the gel with sec-butanol for about 2 hours, removing the overlay and replacing it with water;

(j)   cutting the IPG gel strips to a size suitable for the second dimensional electrophoresis, removing 6 mm from the anode end and 14 mm from the cathode end;

(k) over-layering the slab gel with an aqueous solution of agarose (0.5% w/v) and Tris-glycine-SDS (25 mM-198 mM-0.1% w/v) as leading buffer, heated to 70°C and loading the IPG gel strips onto the slab gel through this over-layered solution;

(l)   running the second dimensional electrophoresis at a constant current of 40 mA at 8-12°C for 5 hours; and

(m) washing the gel.

50. The protein of claim 49, wherein the protein is selected from MOM 31, MOM 32, MOM 34, MOM 36, MOM 33, MOM 35, WOM 37, WOM 52, WOM 60, WOM 38, WOM 53, WOM 61, WOM 44, WOM 54, WOM 62, WOM 45, WOM 55, WOM 63, WOM 48, WOM 57, WOM 64, WOM 49, WOM 58, WOM 50, WOM 59, WOM 39, WOM 40, WOM 41, WOM 42, WOM 43, WOM 46, WOM 47, WOM 51, BOM 66, BOM 67, BOM 68, BOM 75, BOM 76, BOM 77, BOM 69, BOM 70, BOM 71, BOM 72, BOM 73, BOM 74.

51. A differentially expressed protein having one or more of the identifying characteristics as set out any one of Tables 3 to 5.

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52. The protein of claim 51, wherein the identifying
characteristics are pI and Mw.

5

Figure 1A

A

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Figure 1 B

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Figure 1C

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Figure 1 D

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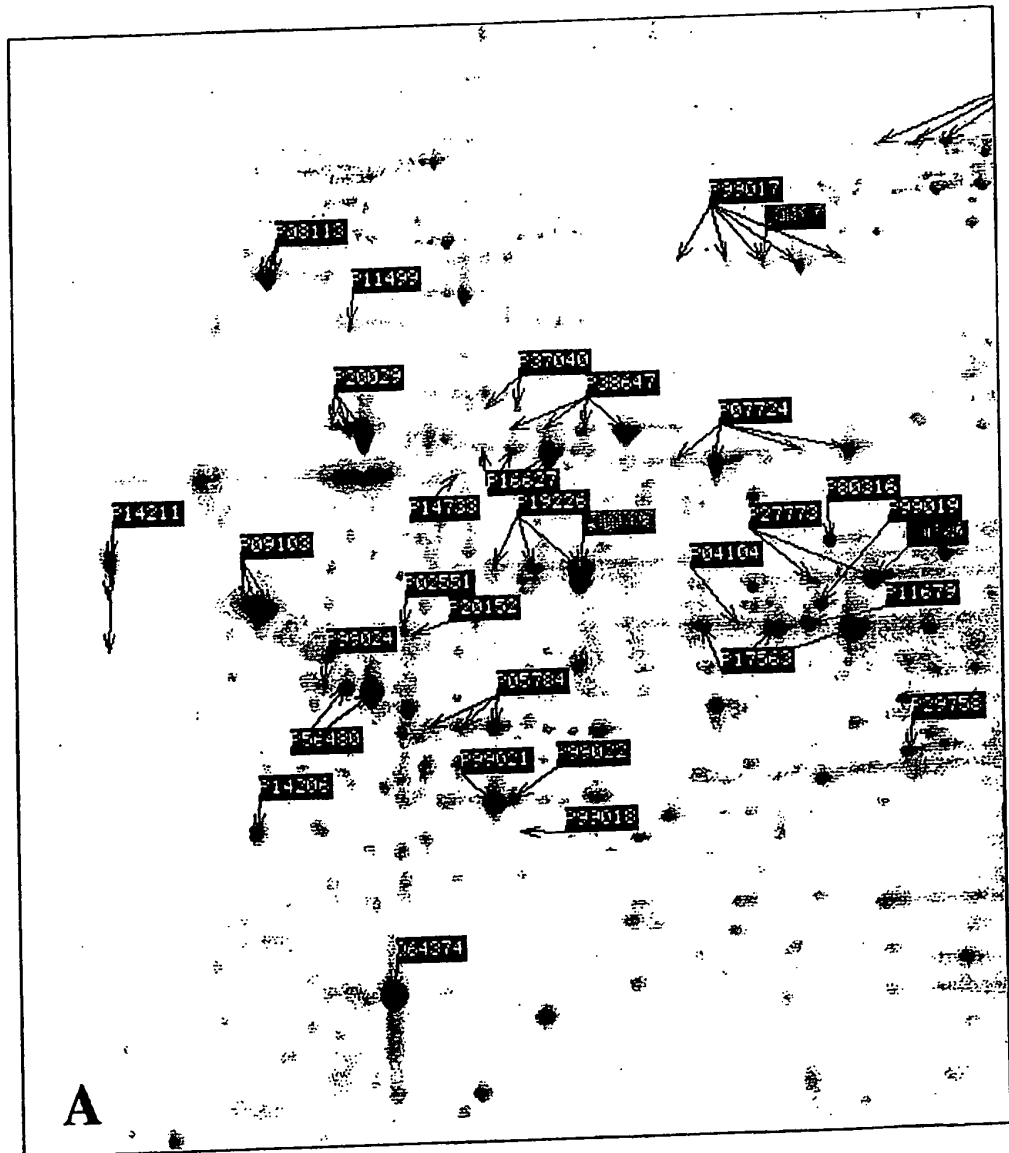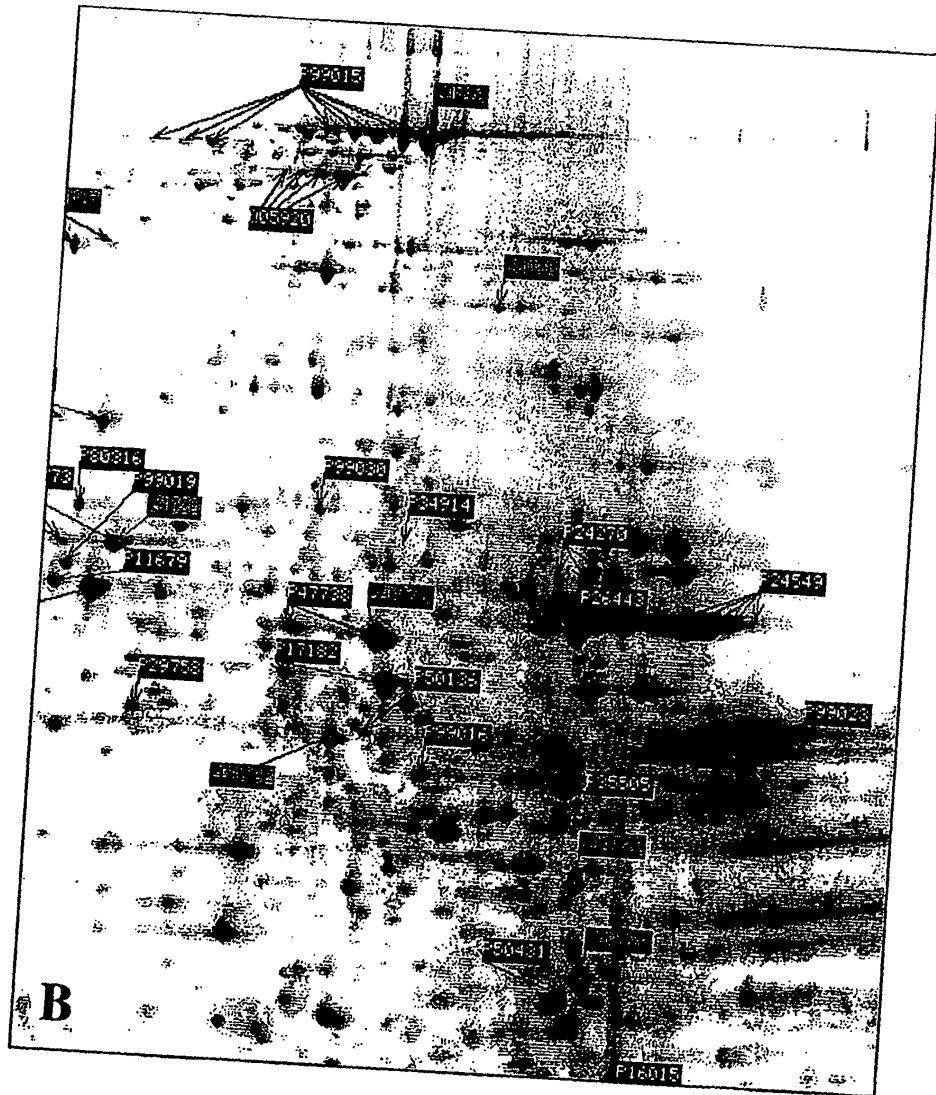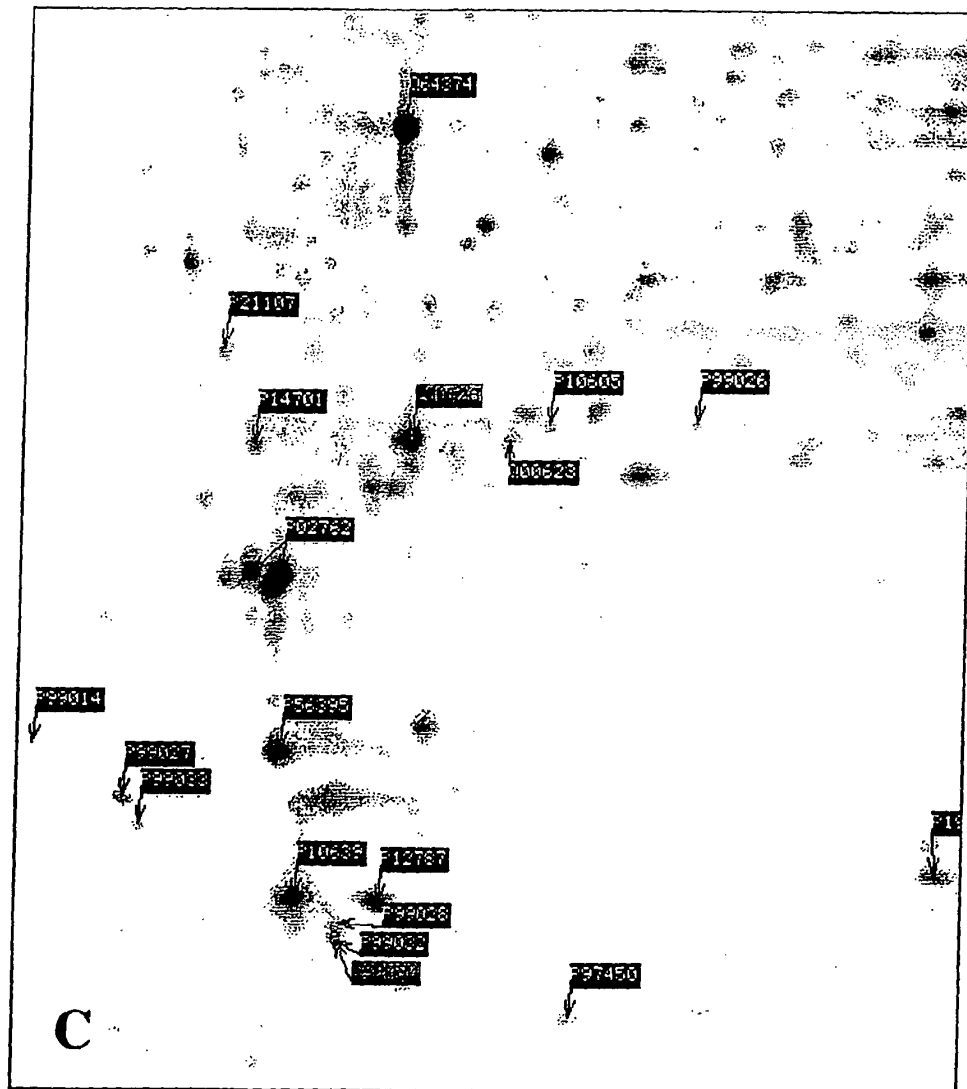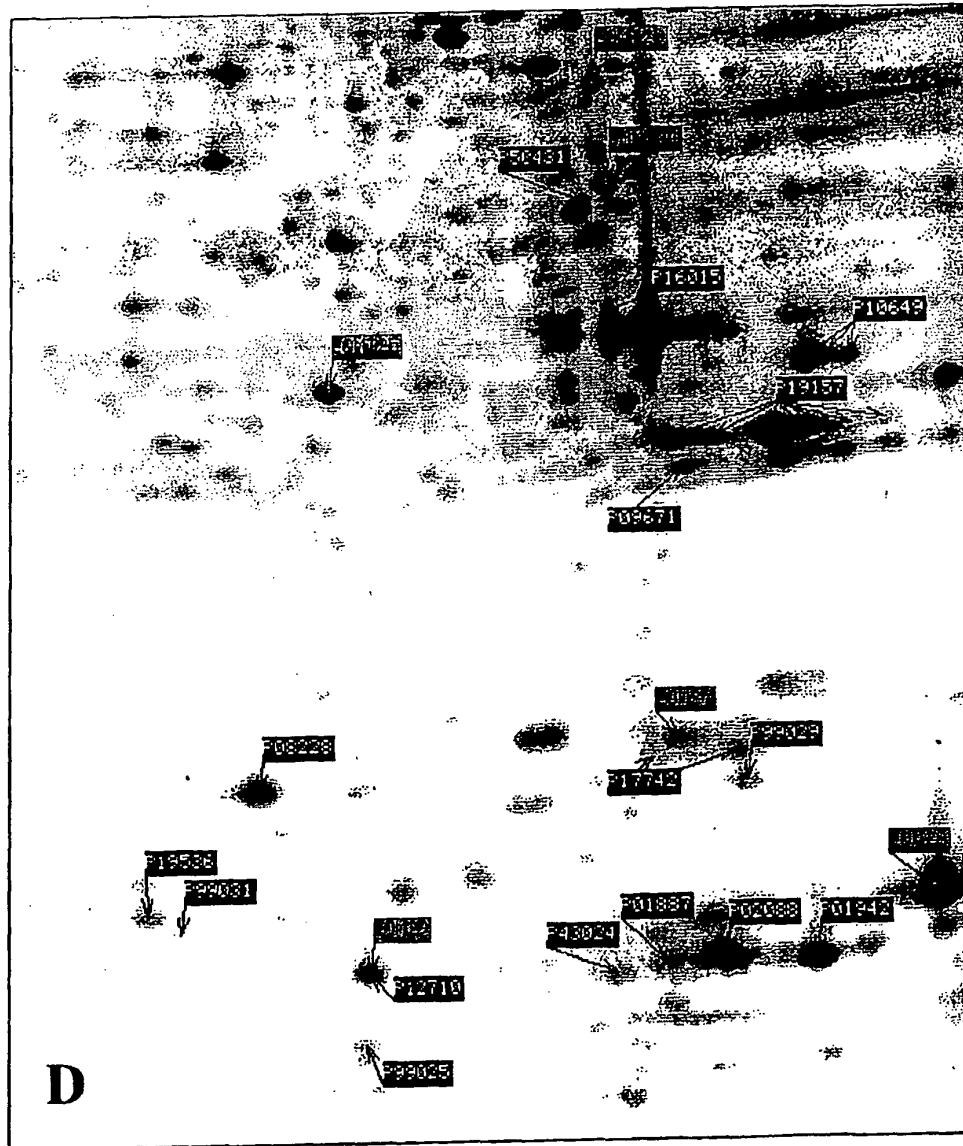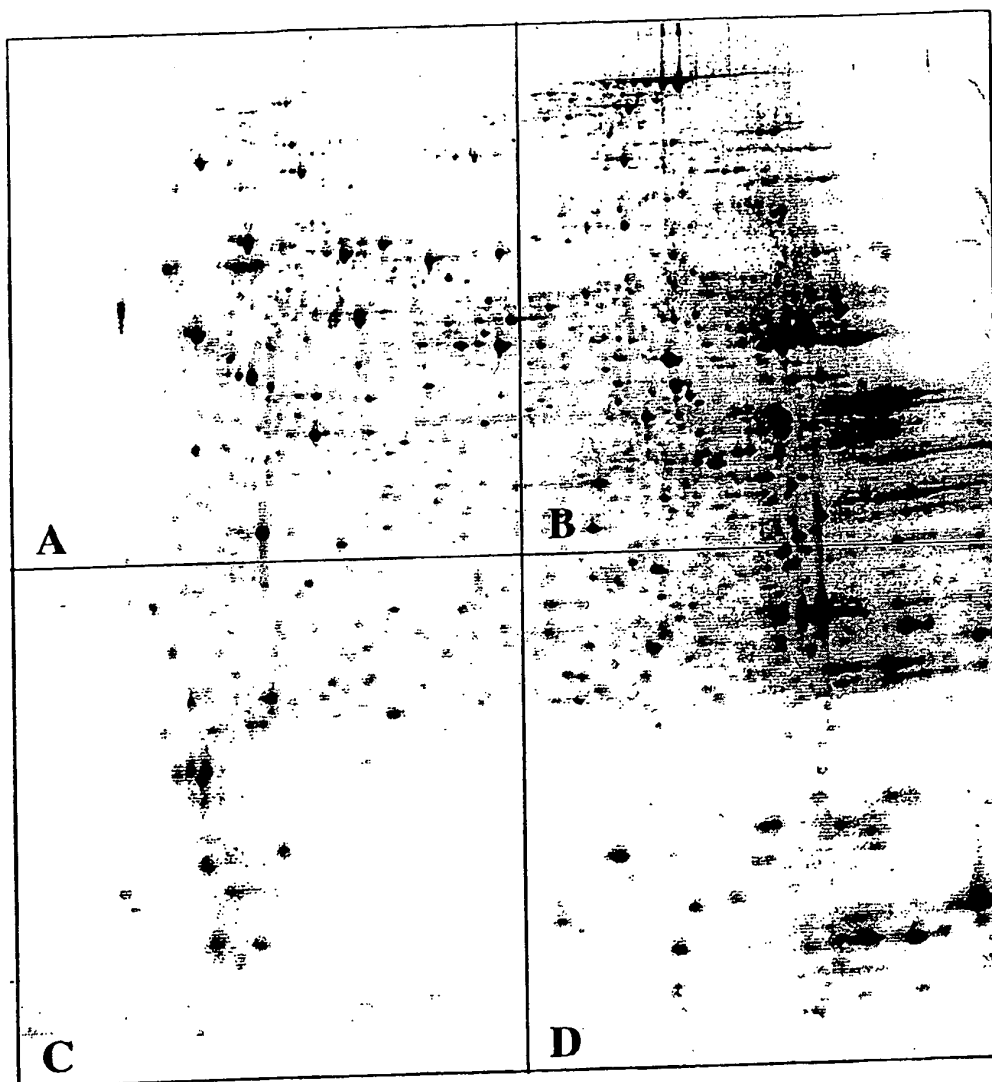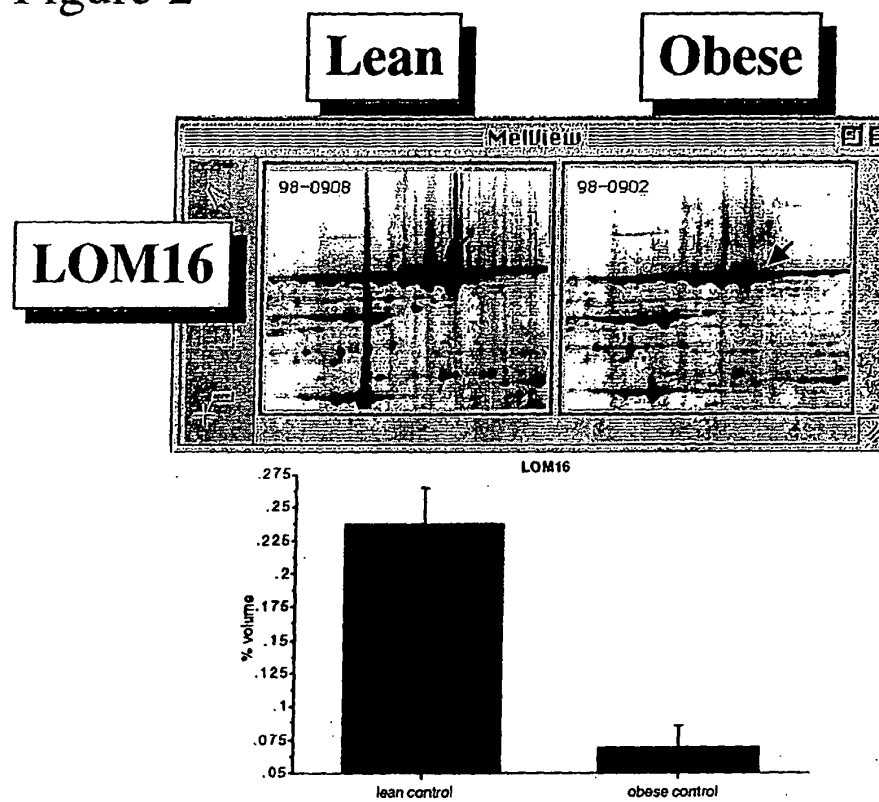
Figure 1E

Figure 2

Figure 2 continued | Lean | | Obese |

LOM17
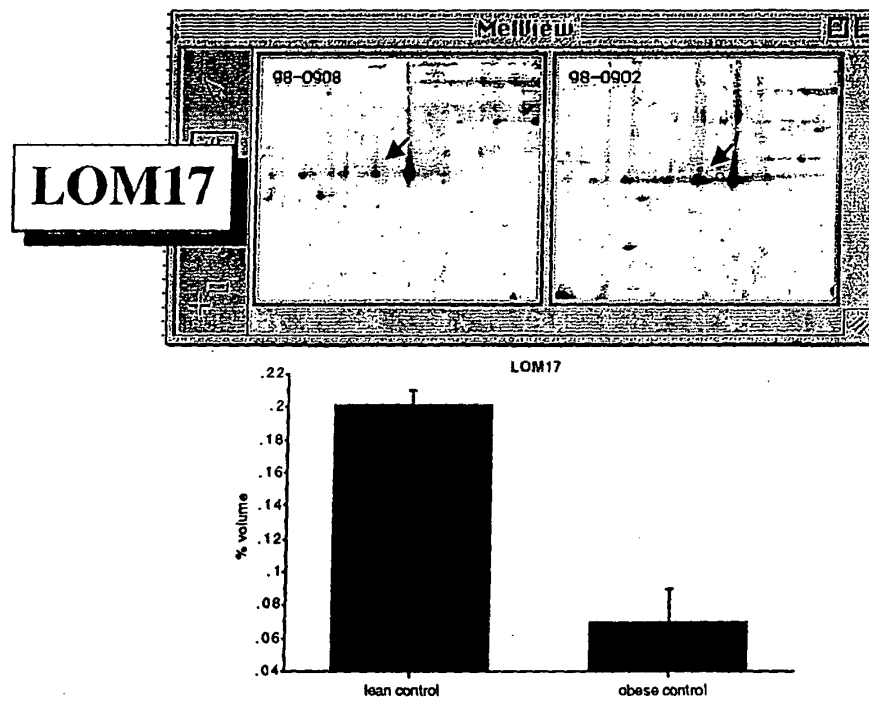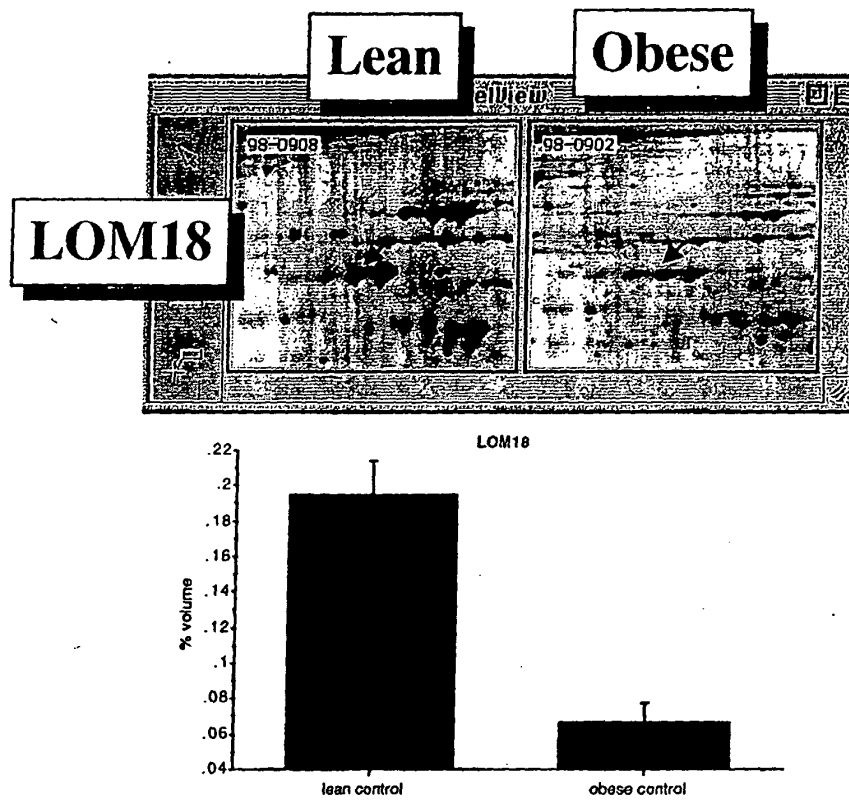
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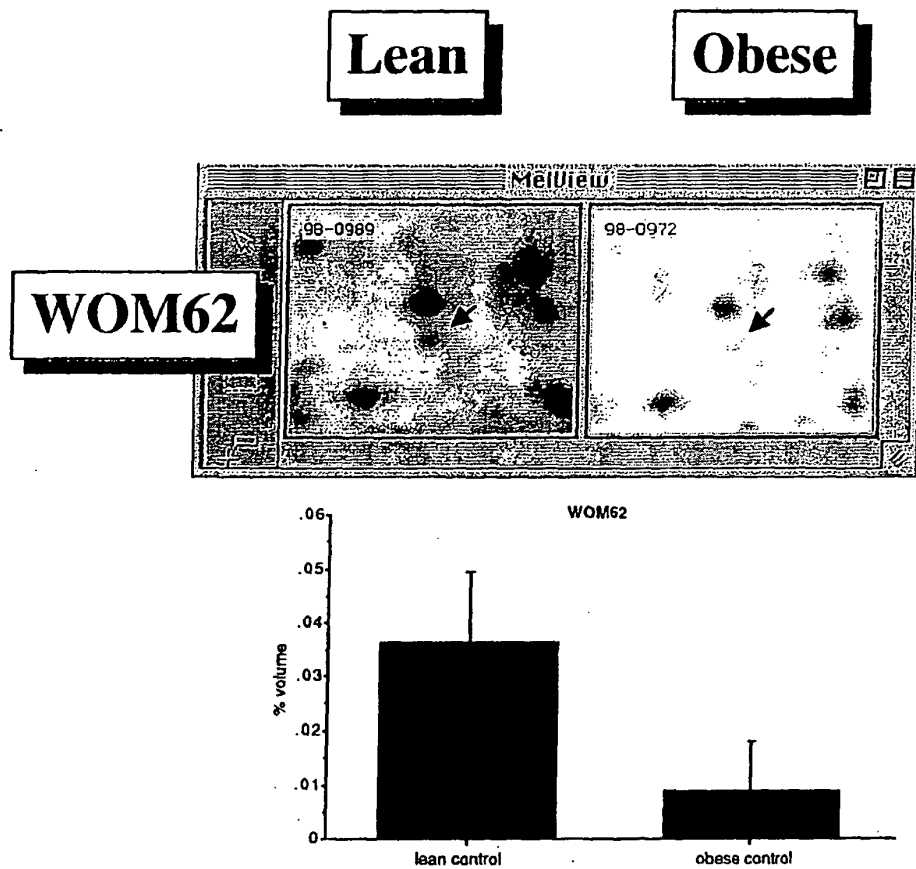
Figure 3 continued

Lean   **Obese**

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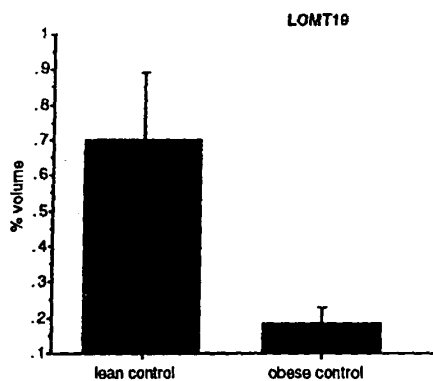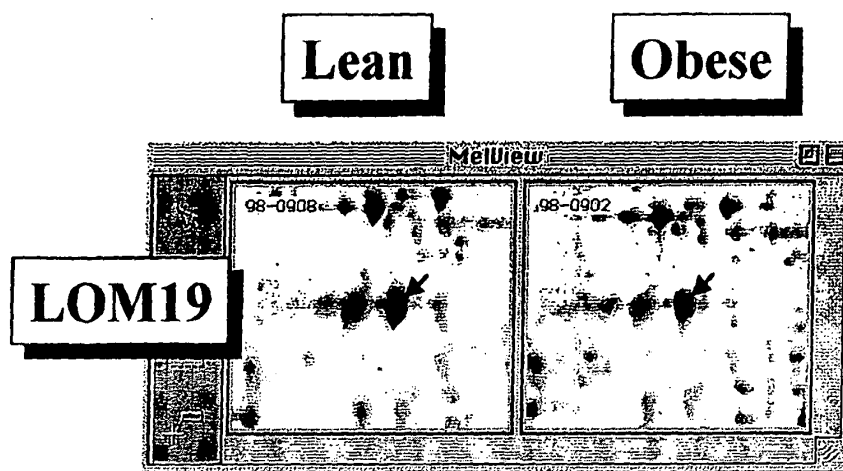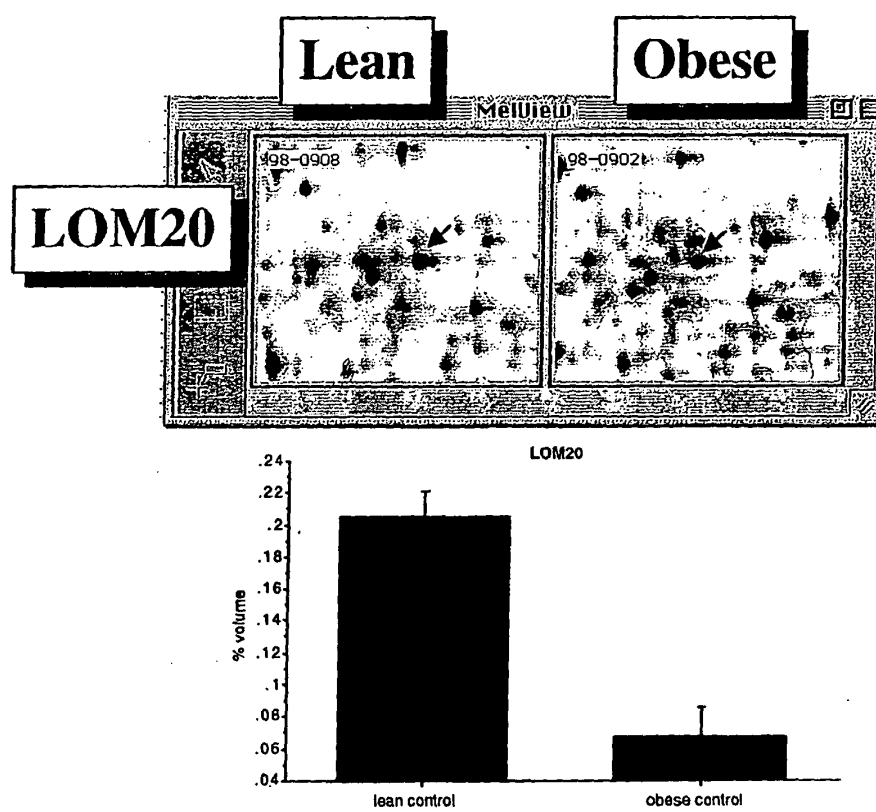
Figure 4

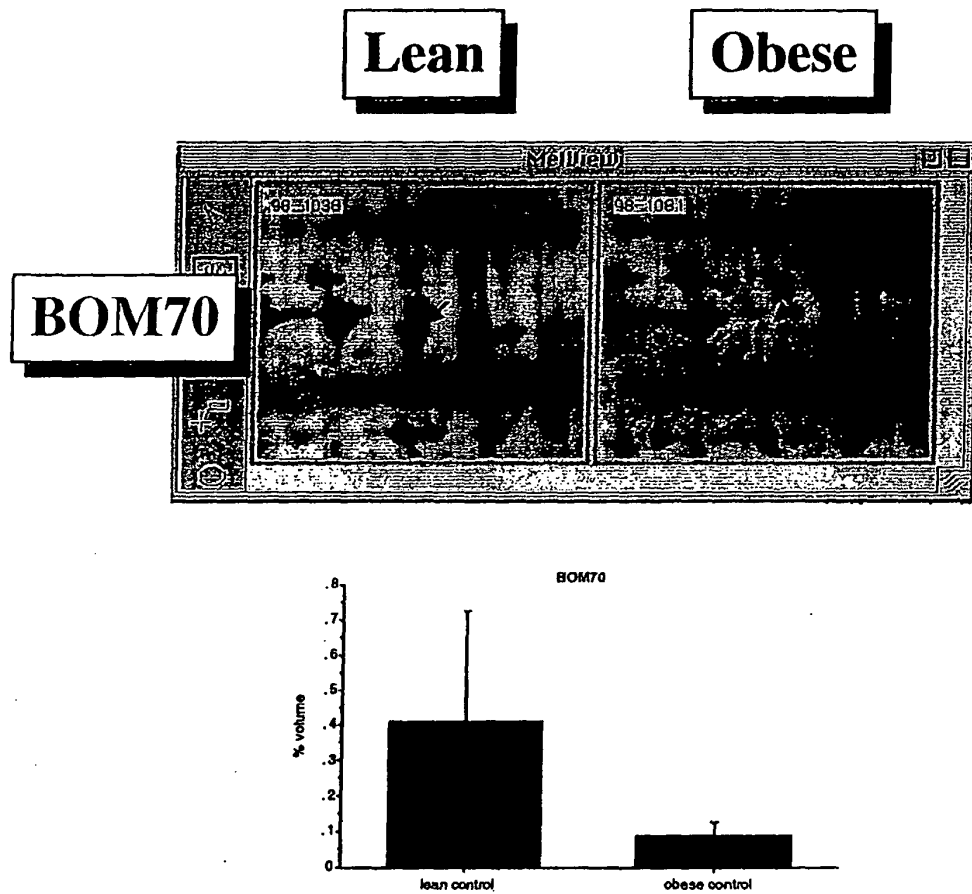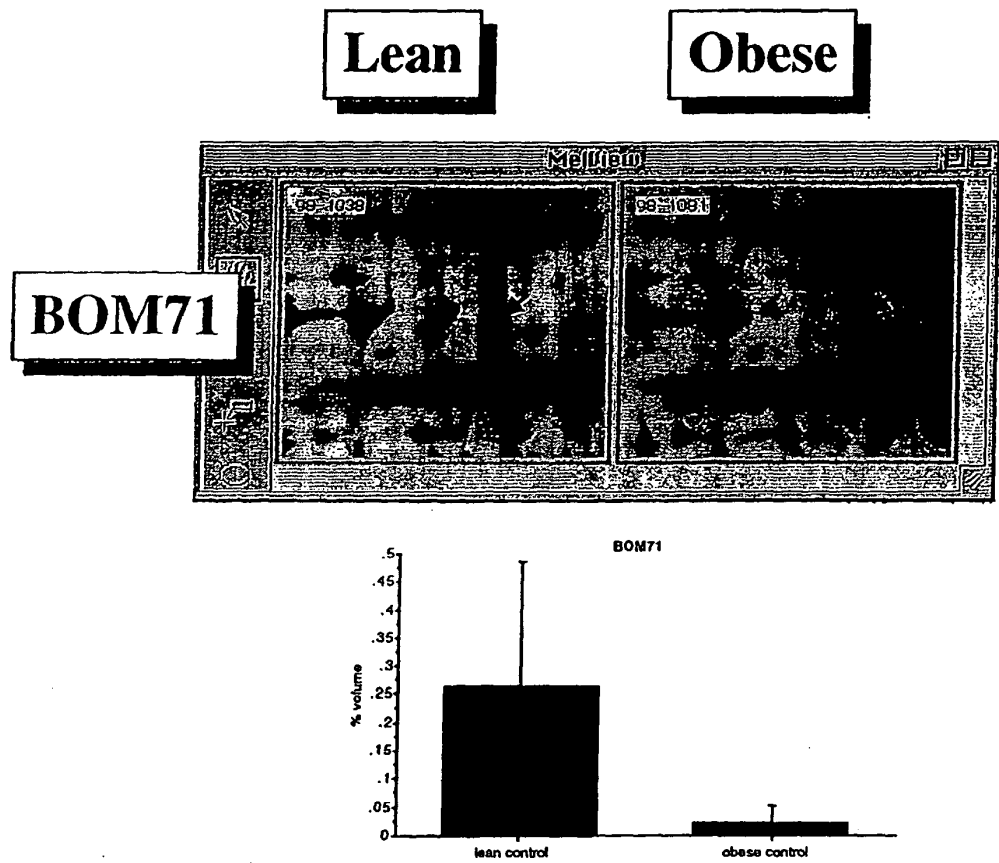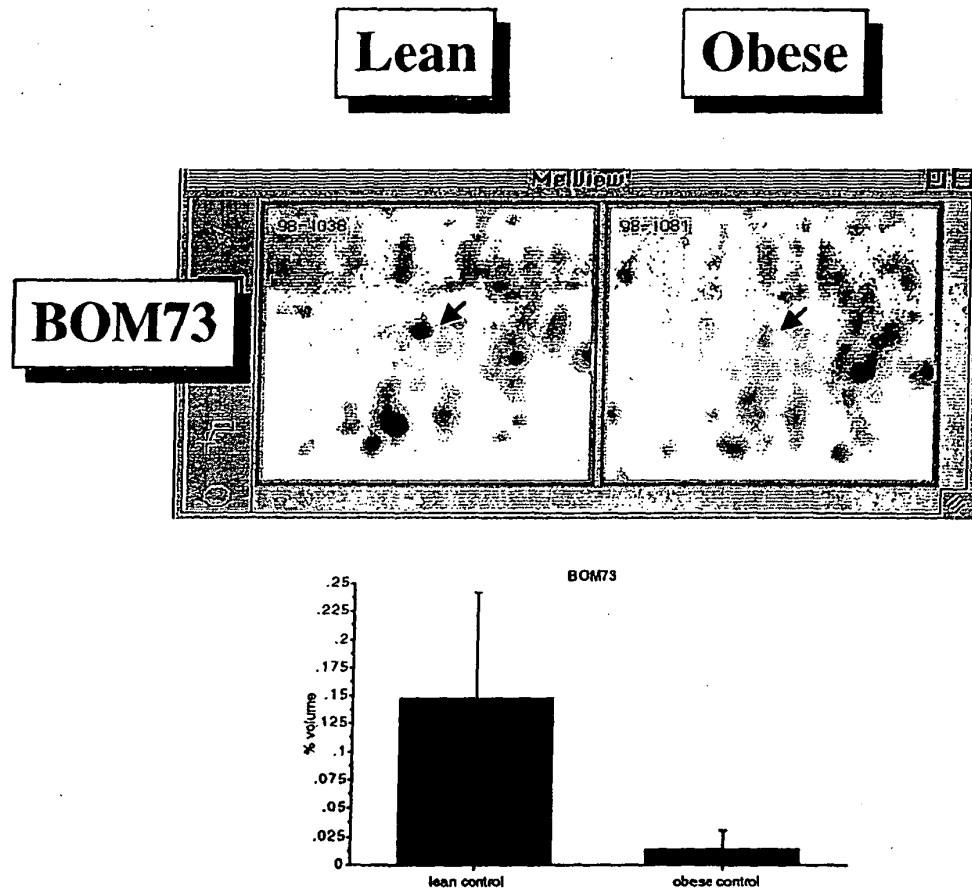# Figure 4 continued
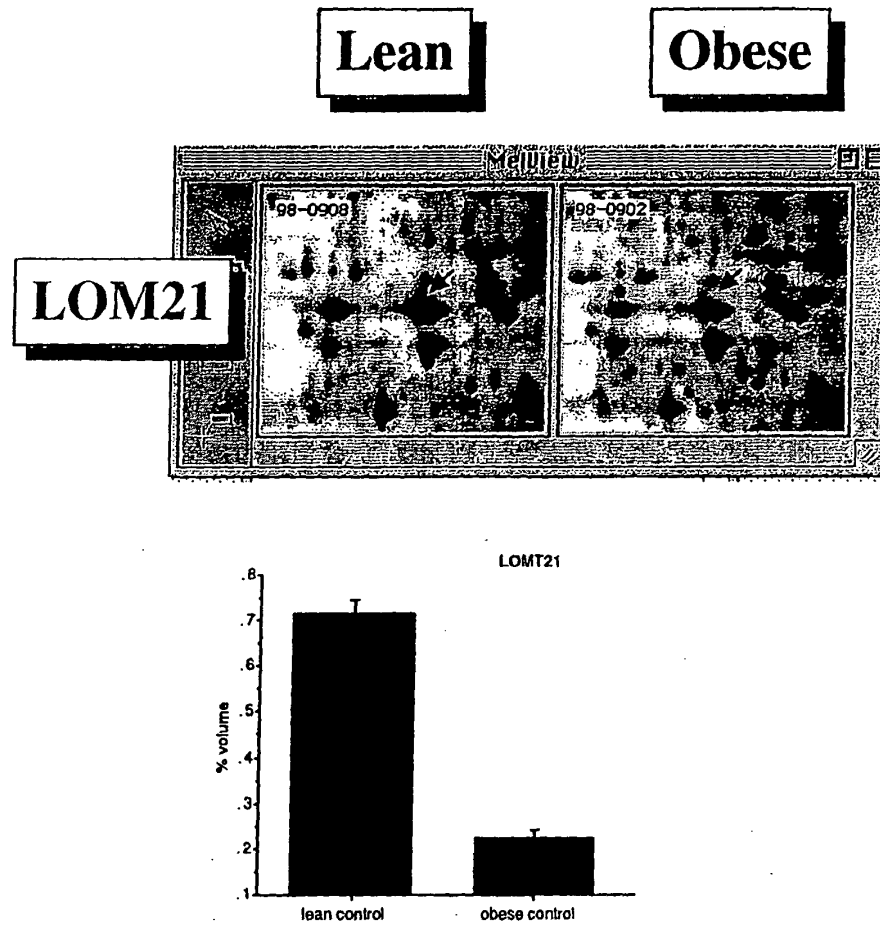
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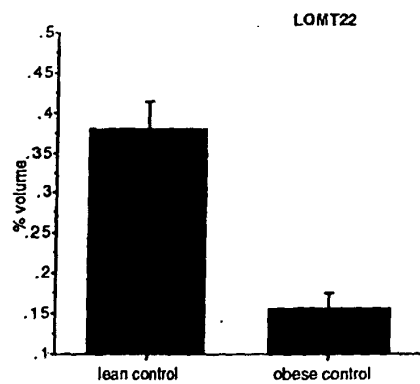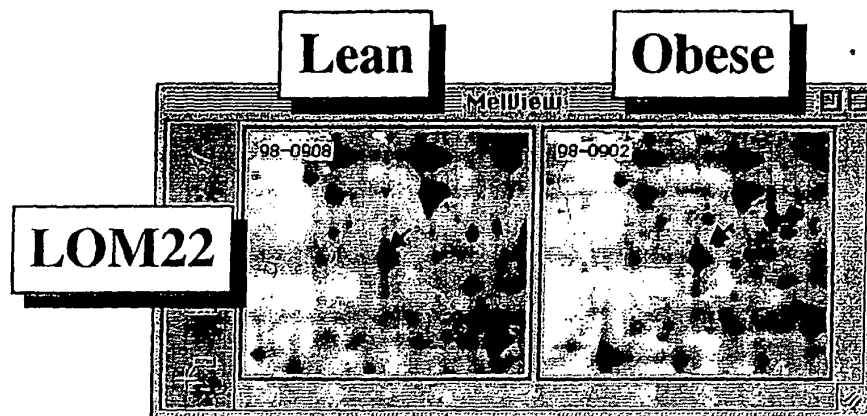
Figure 5 continued

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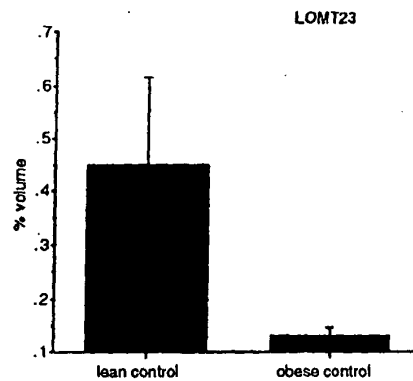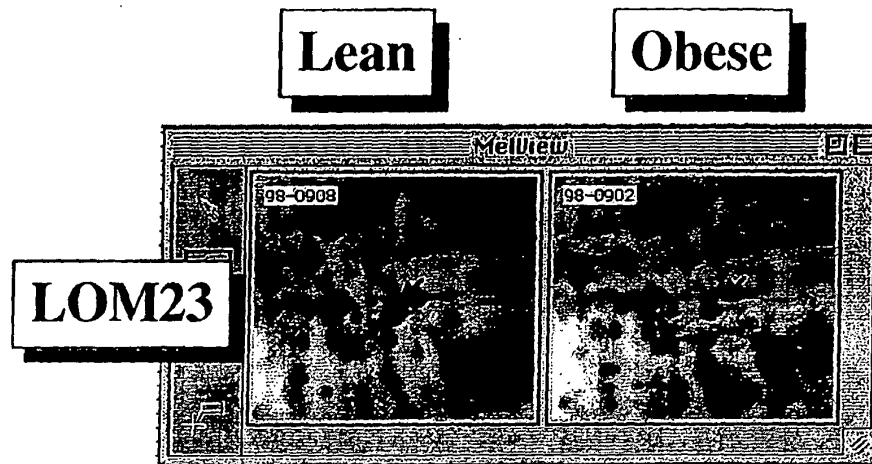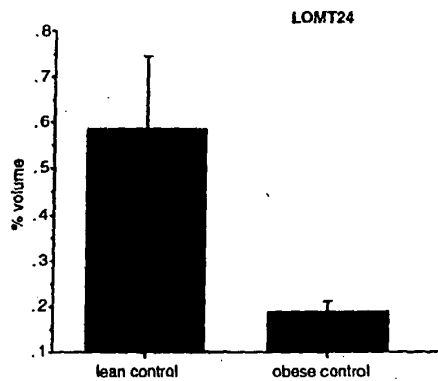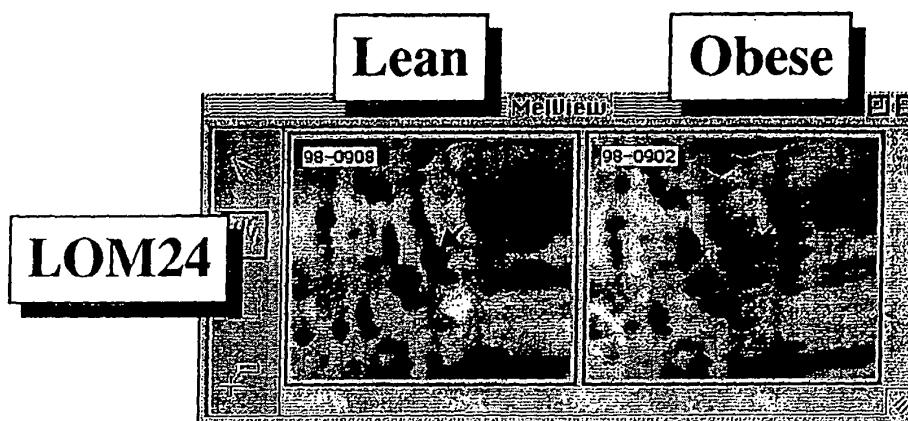
Figure 6

Figure 6 continued
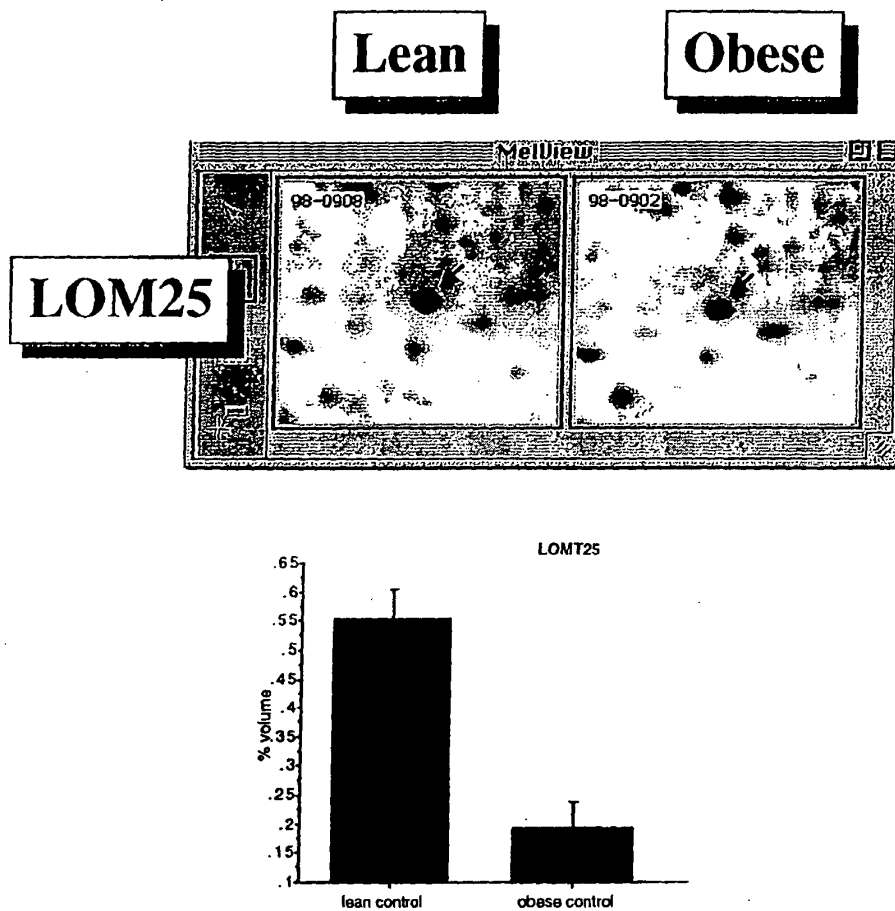
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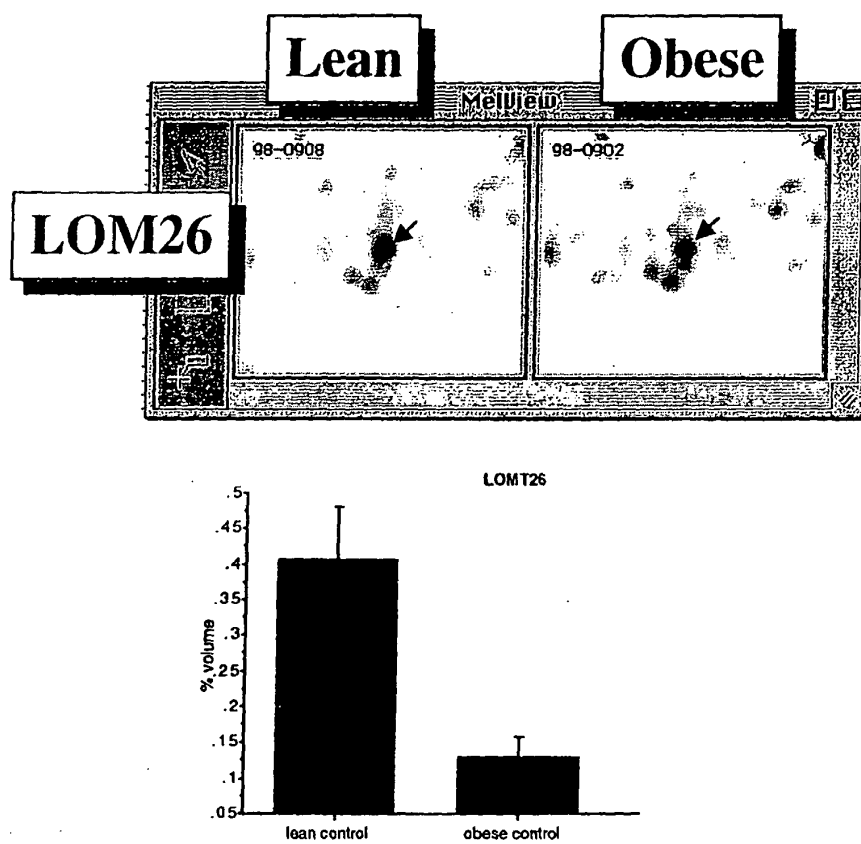
Figure 7 continued

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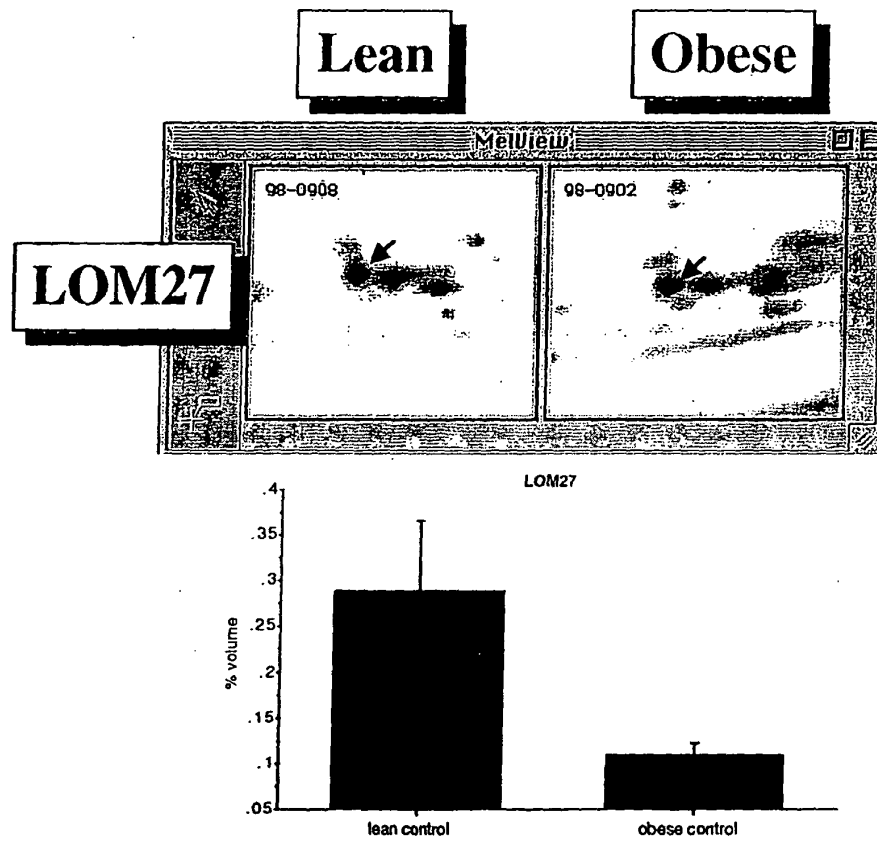
Figure 8

Figure 8 continued

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Figure 9

Lean   Obese

MOM31

MOM32

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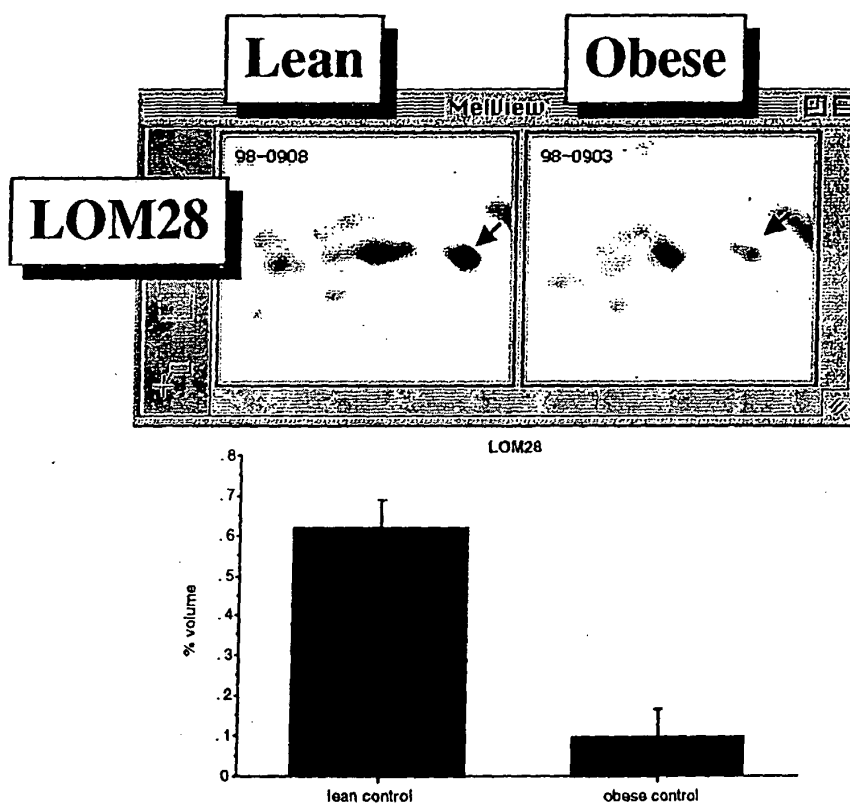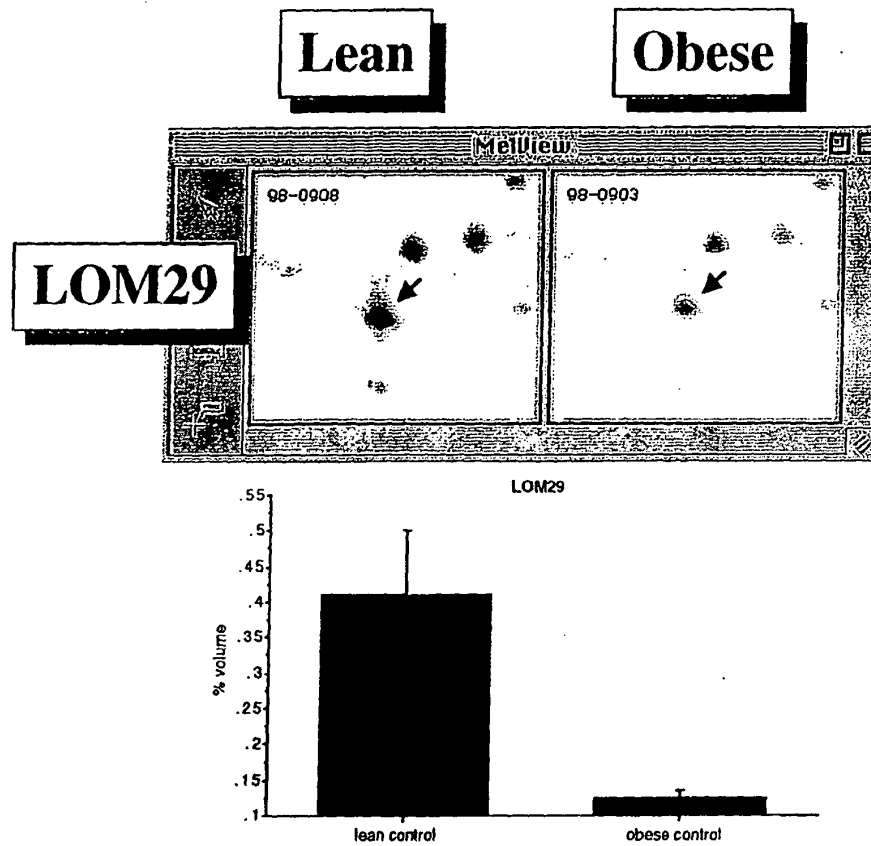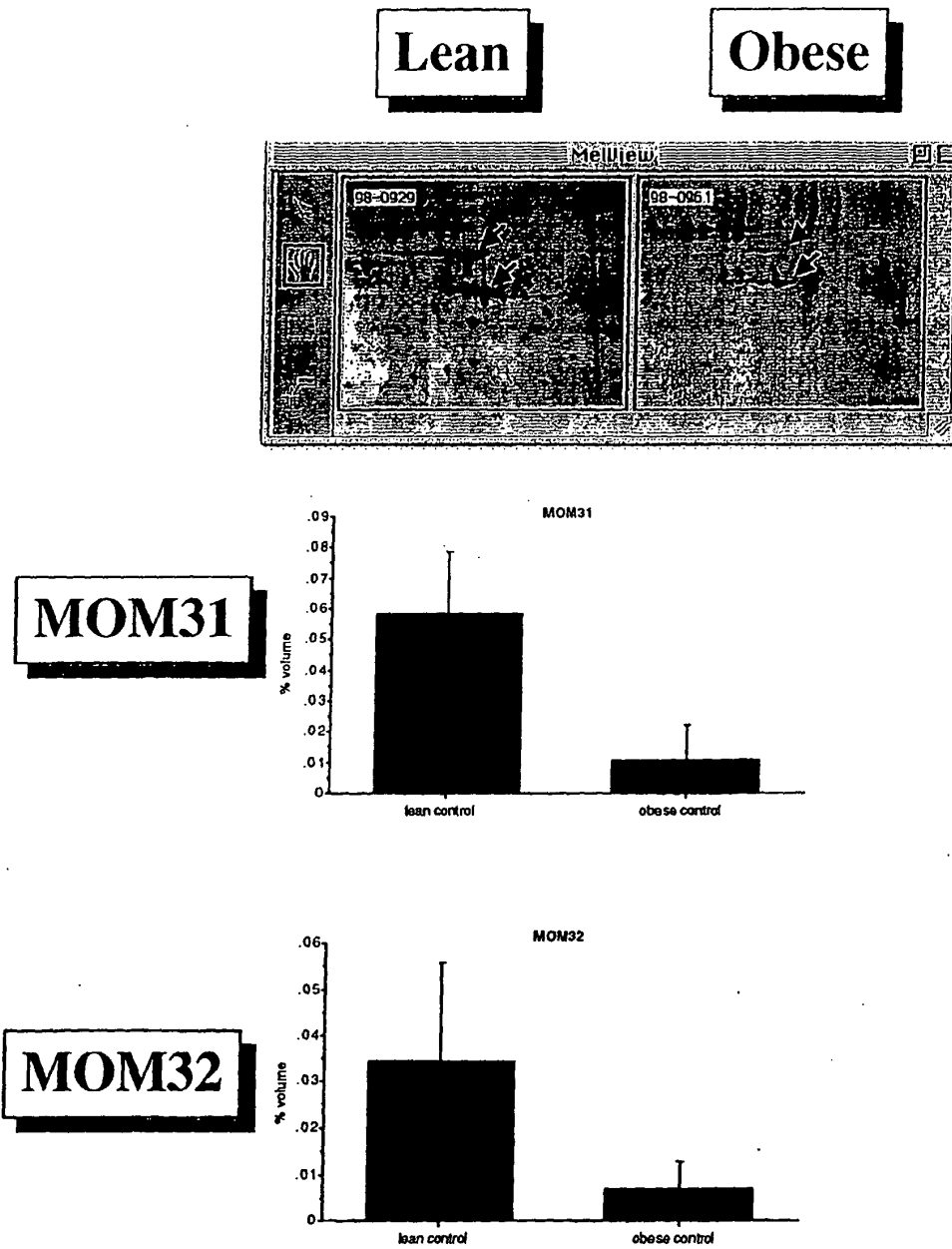
Figure 9 continued

Lean   **Obese**

MOM33
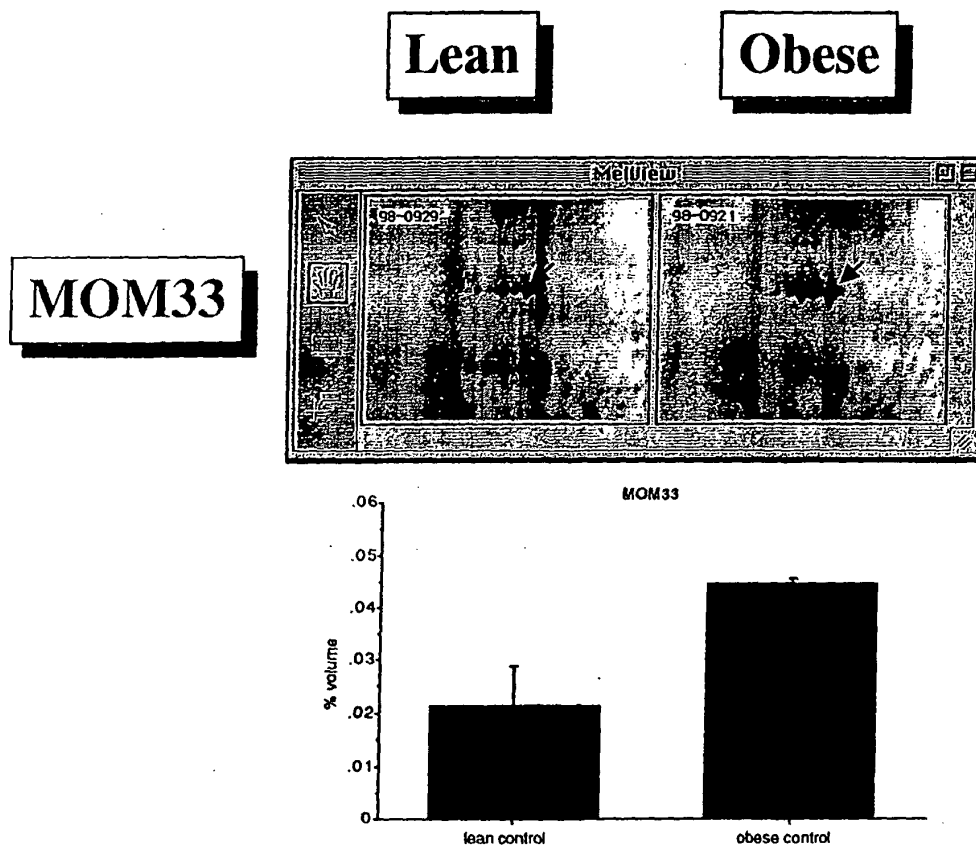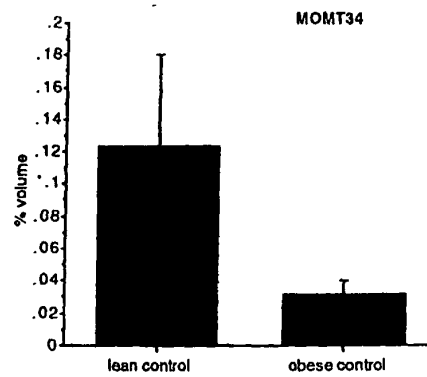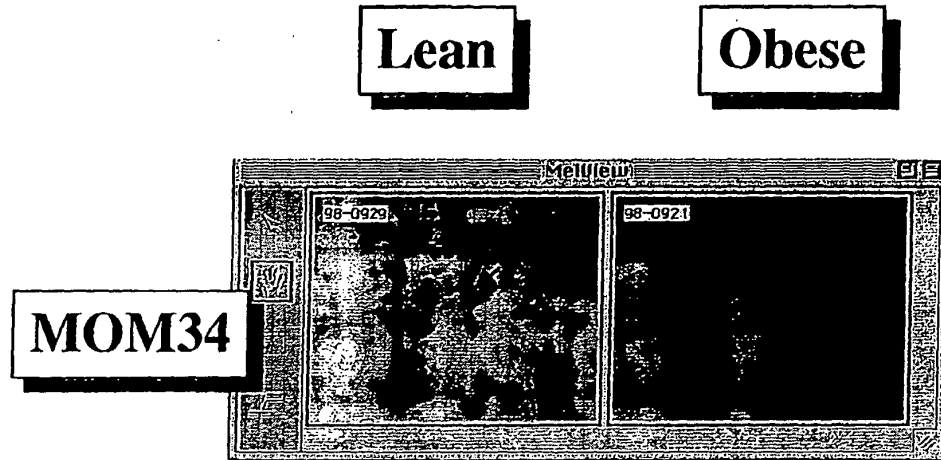
Figure 10

Figure 10 continued
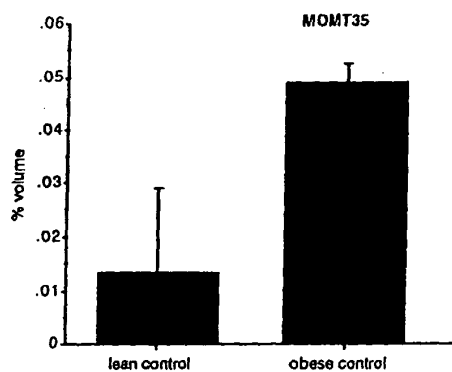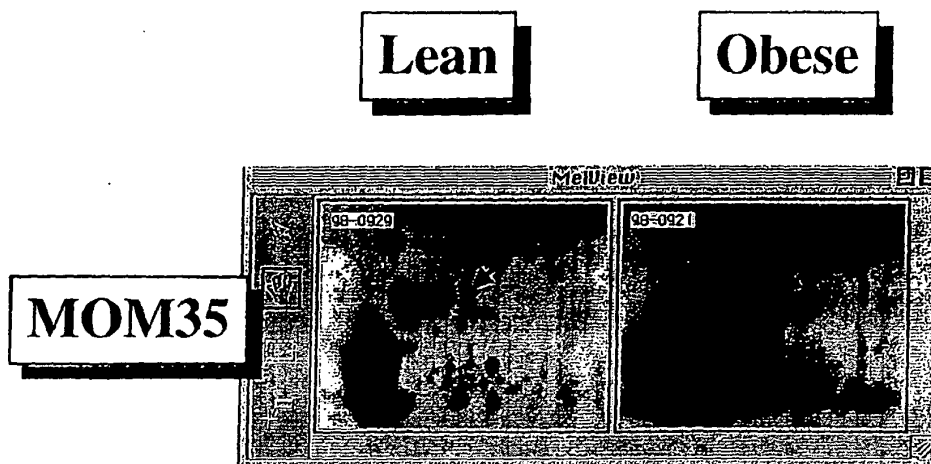
Figure 11

Figure 12

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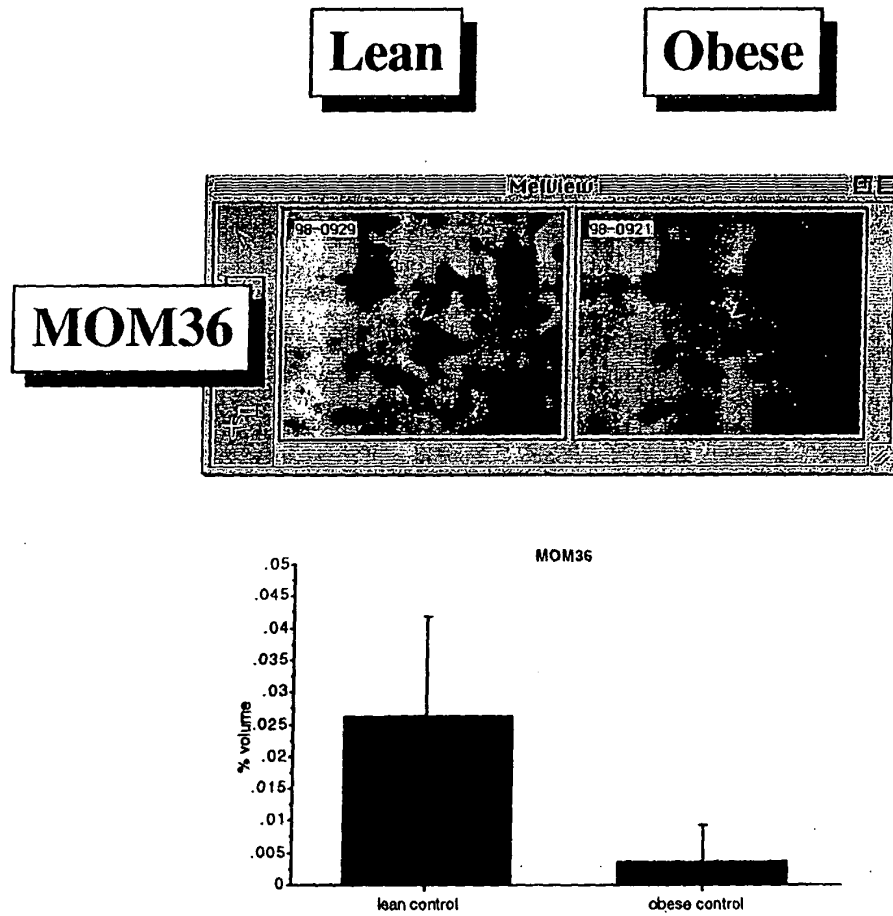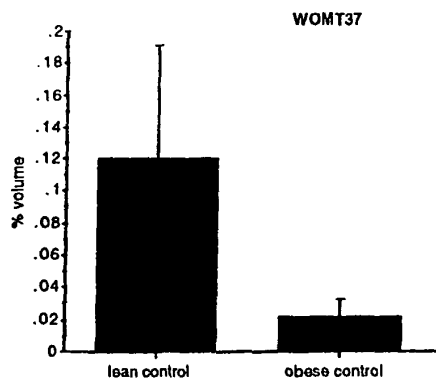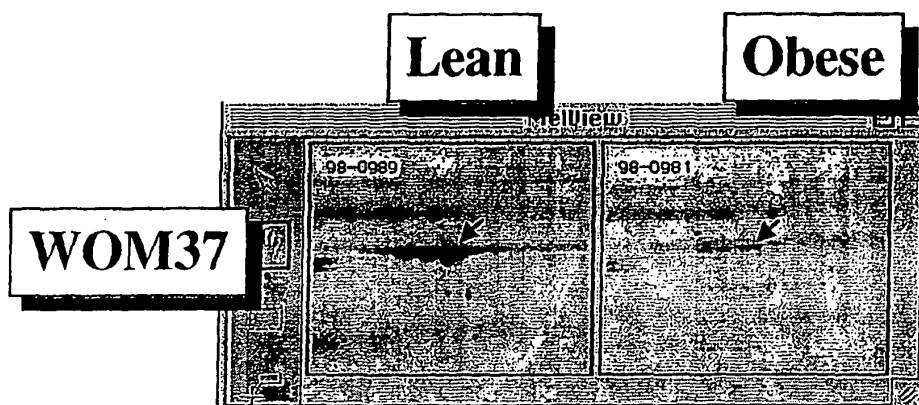
Figure 12 continued

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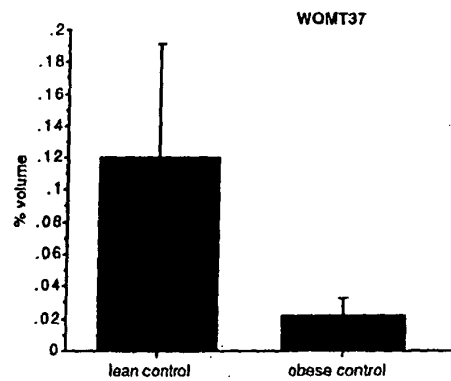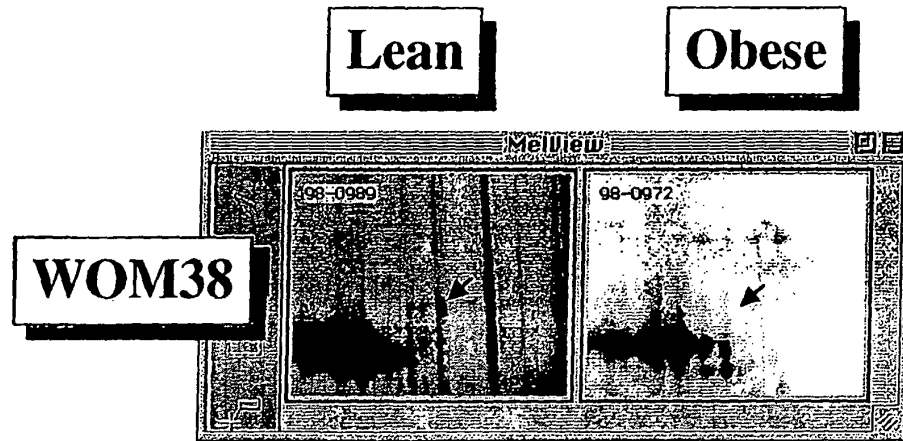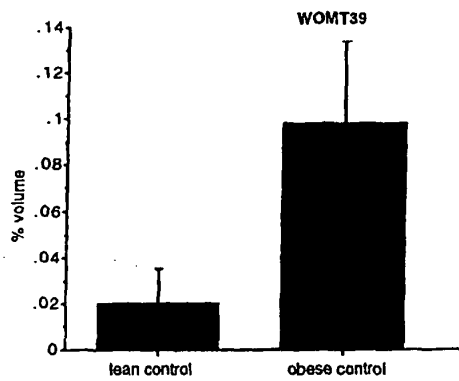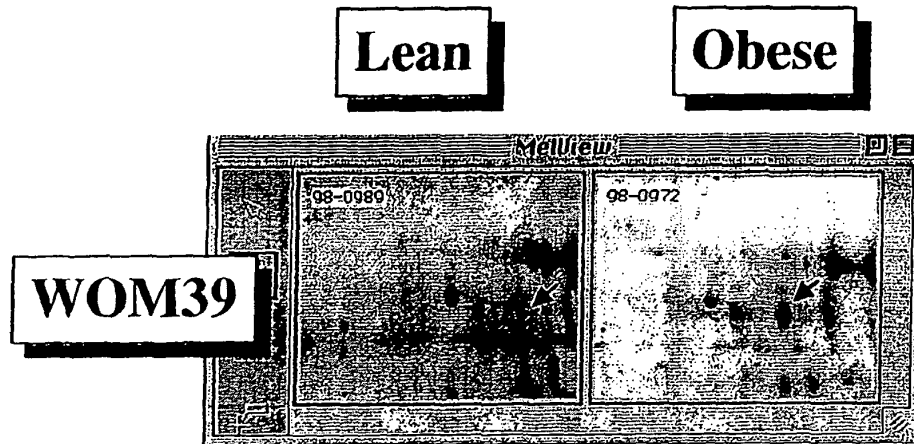
Figure 13

Figure 13 continued
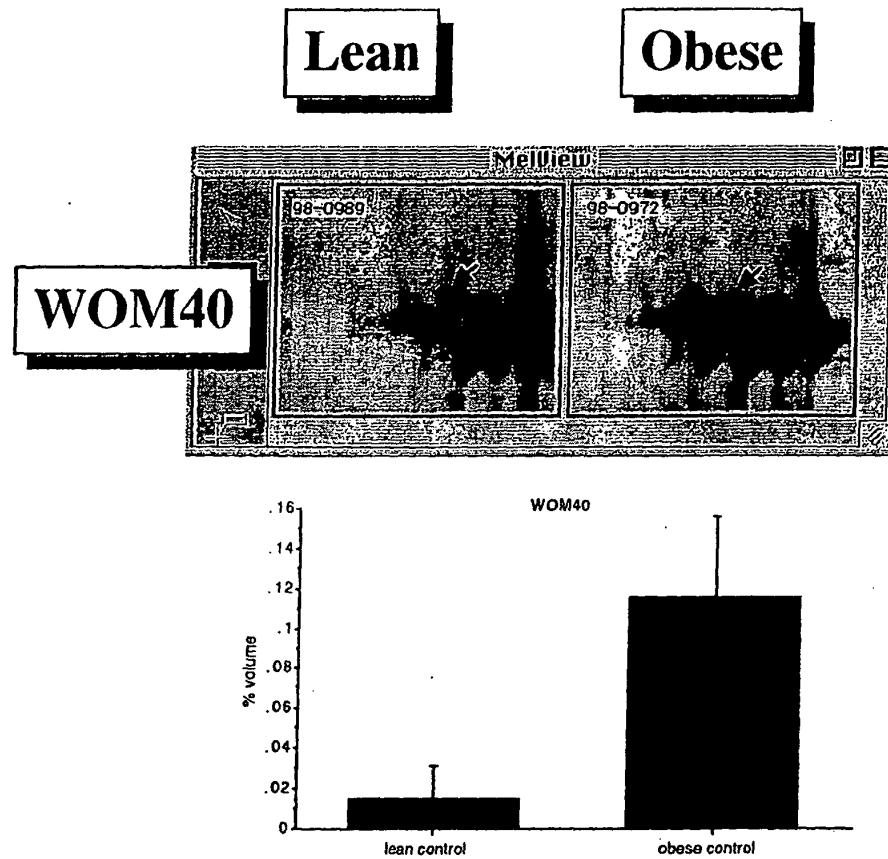
Figure 14
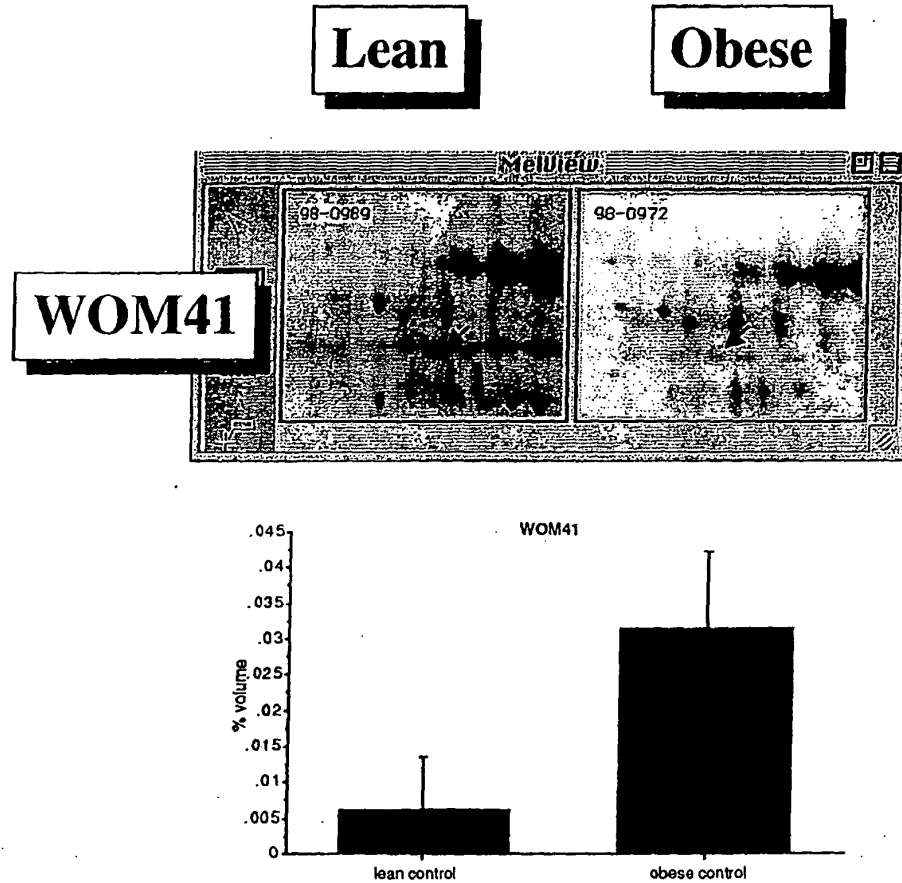
Figure 14 continued

Figure 15

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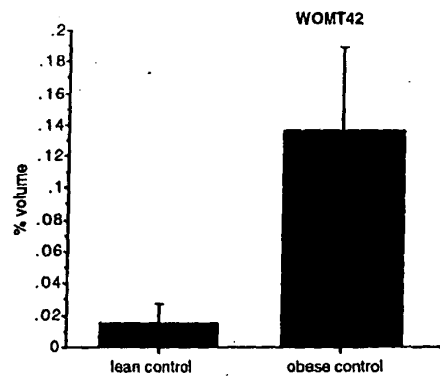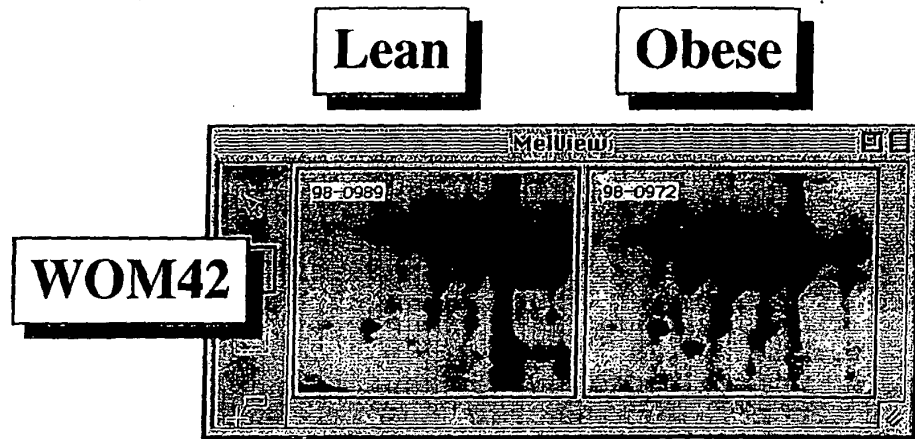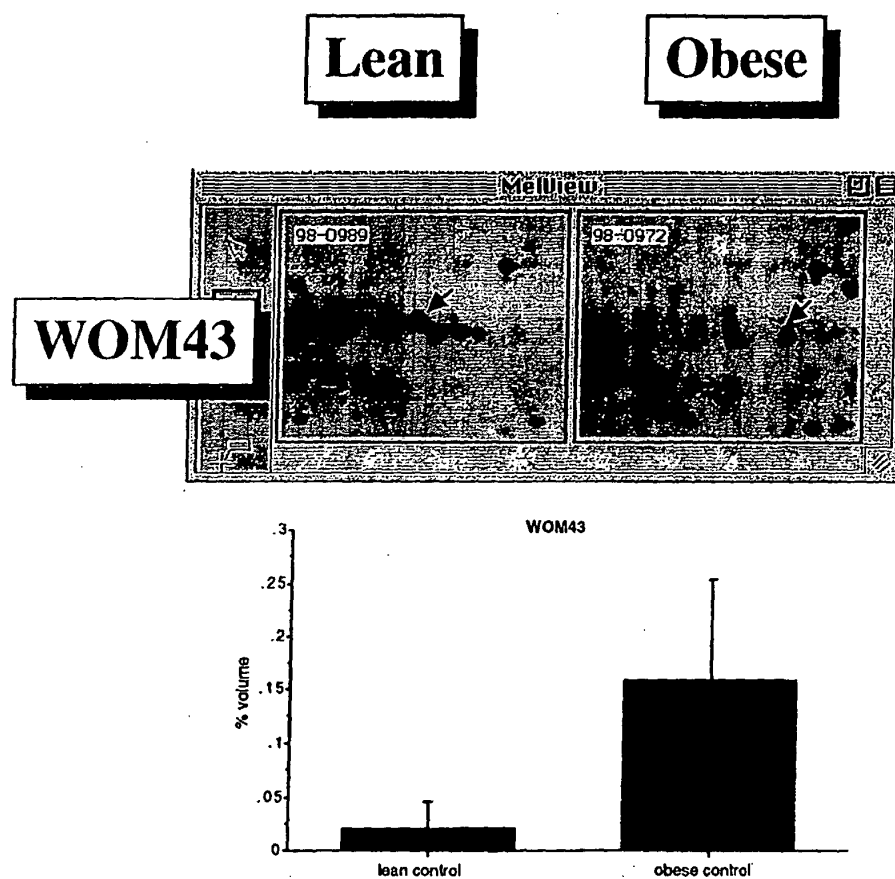
Figure 16   Lean   Obese

WOM46

WOM47

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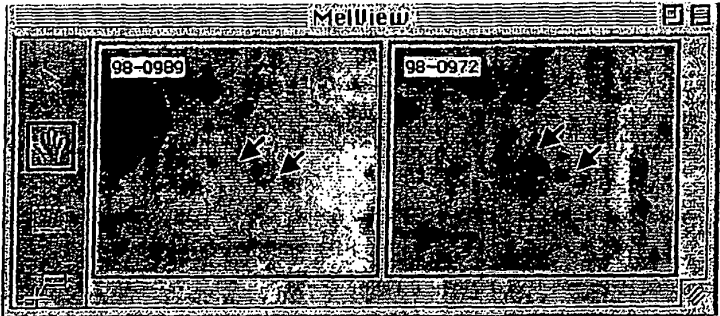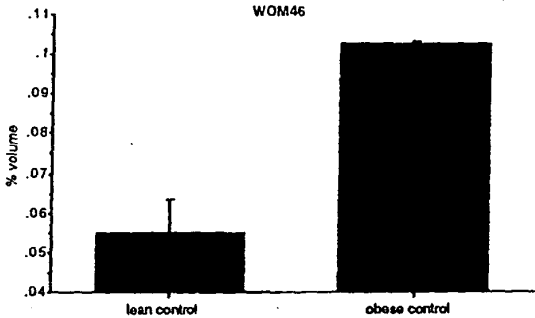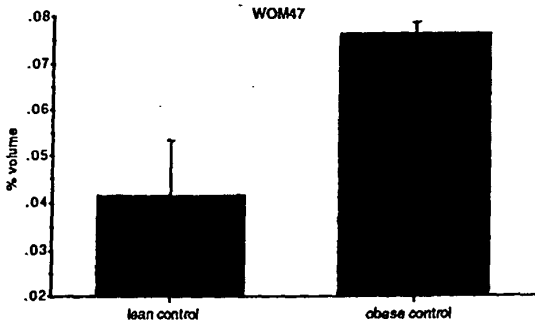
Figure 16 continued

Lean   **Obese**

WOMT48

WOM48

WOMT49

WOM49

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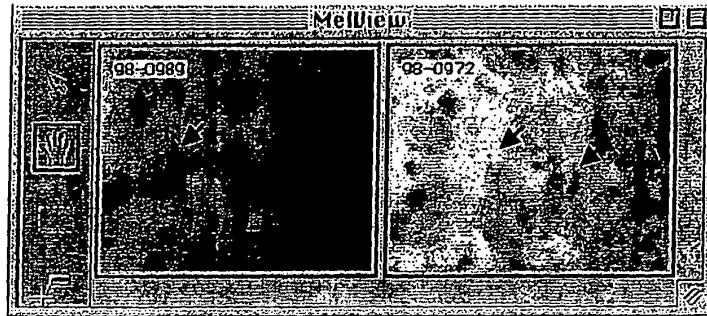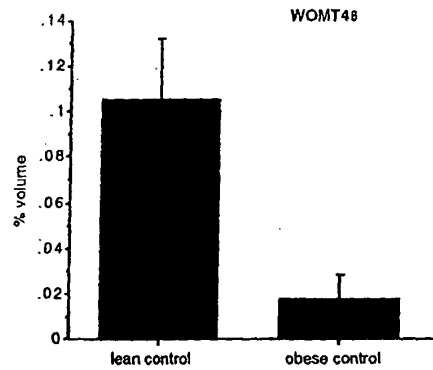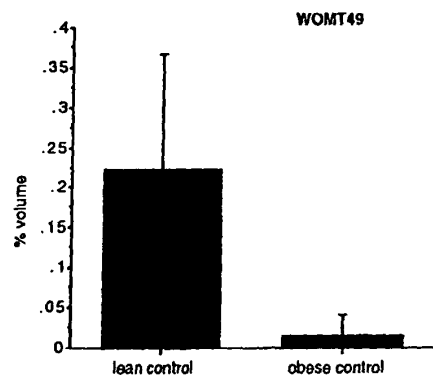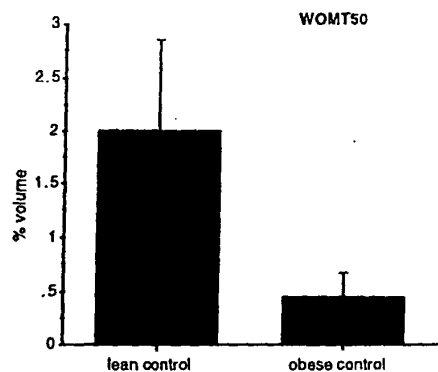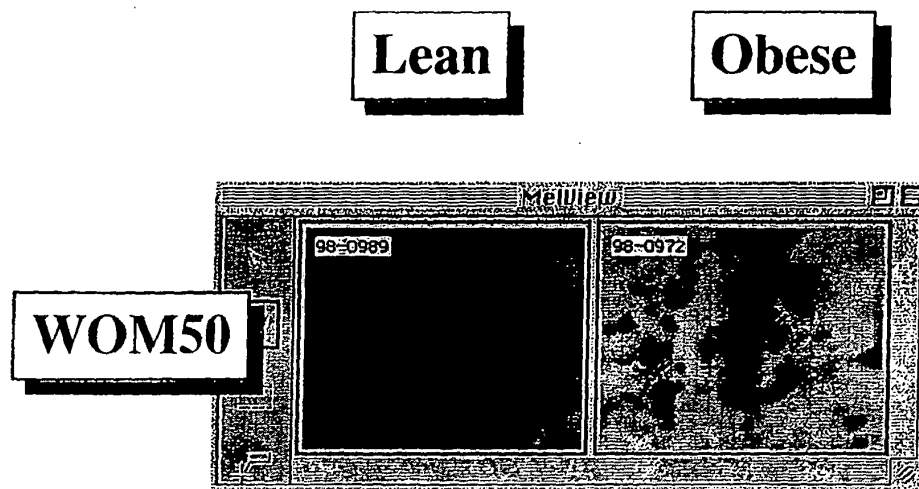
Figure 17

Figure 17 continued

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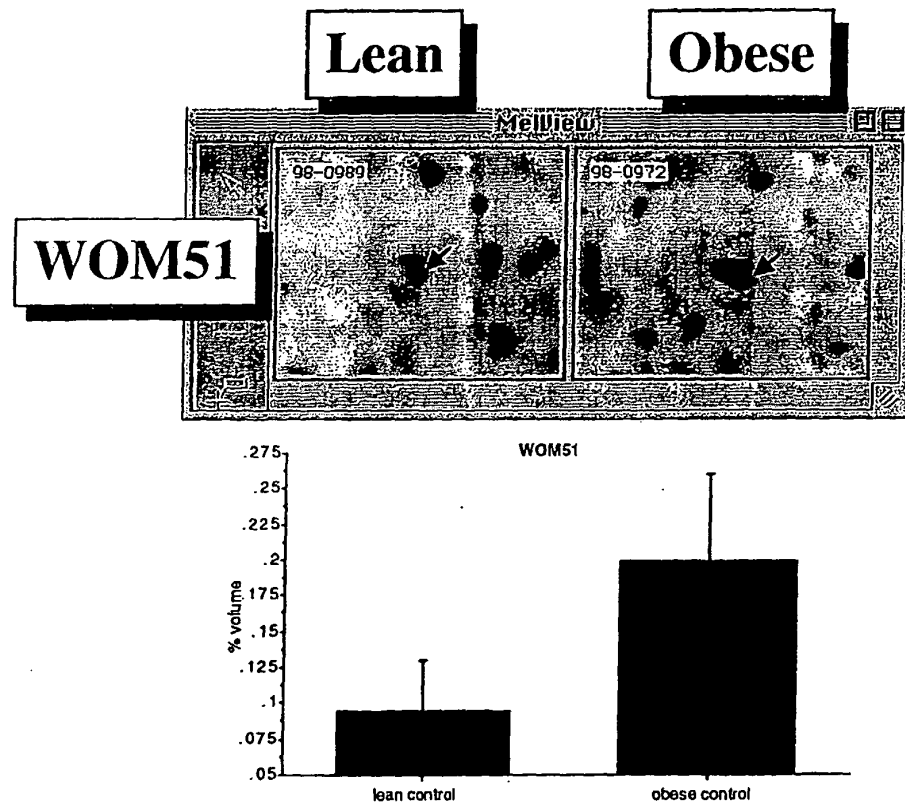
Figure 18

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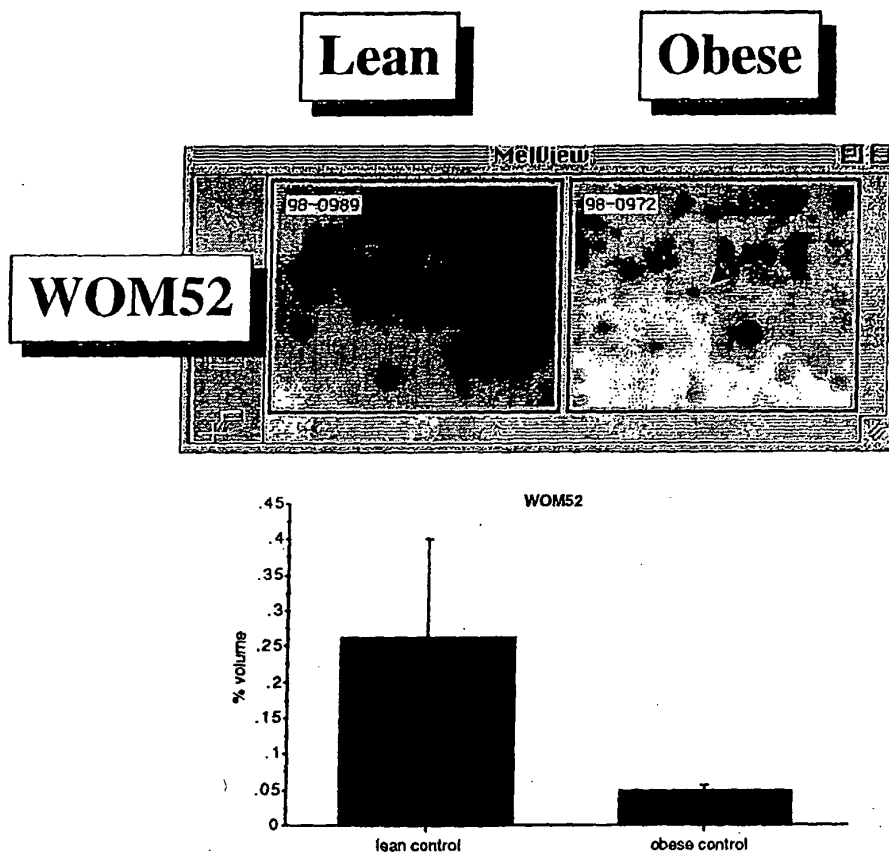
Figure 18 continued
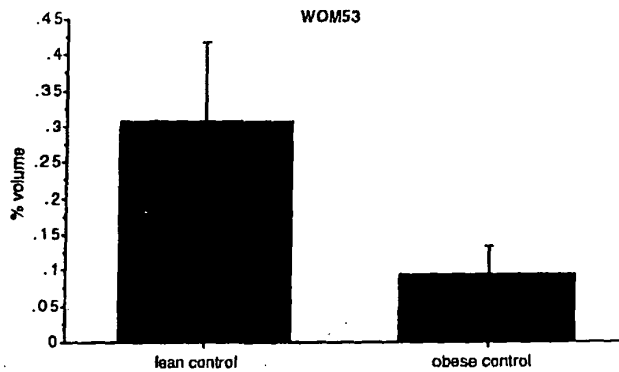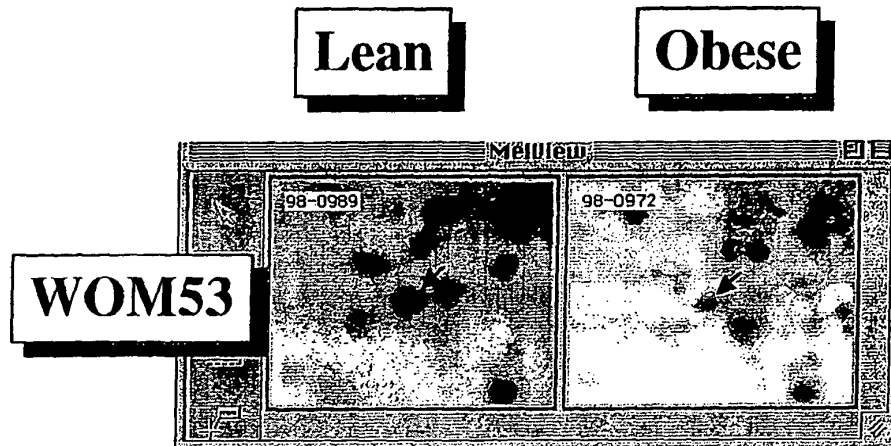
Figure 19
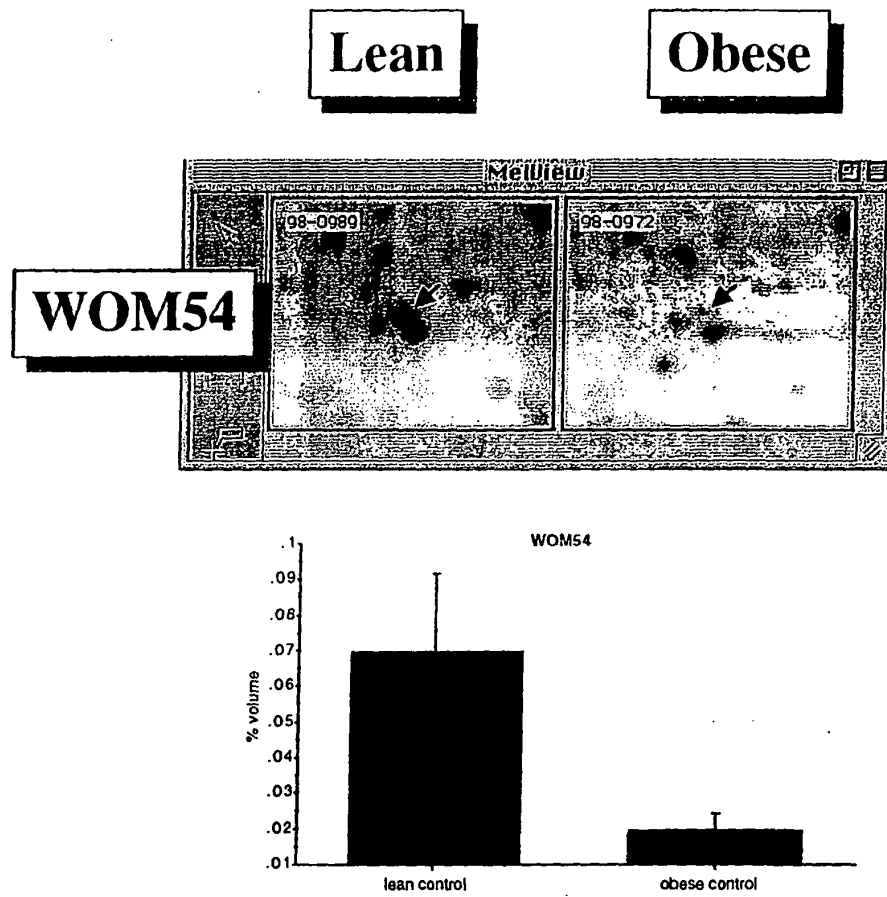
Figure 19 continued

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Figure 20

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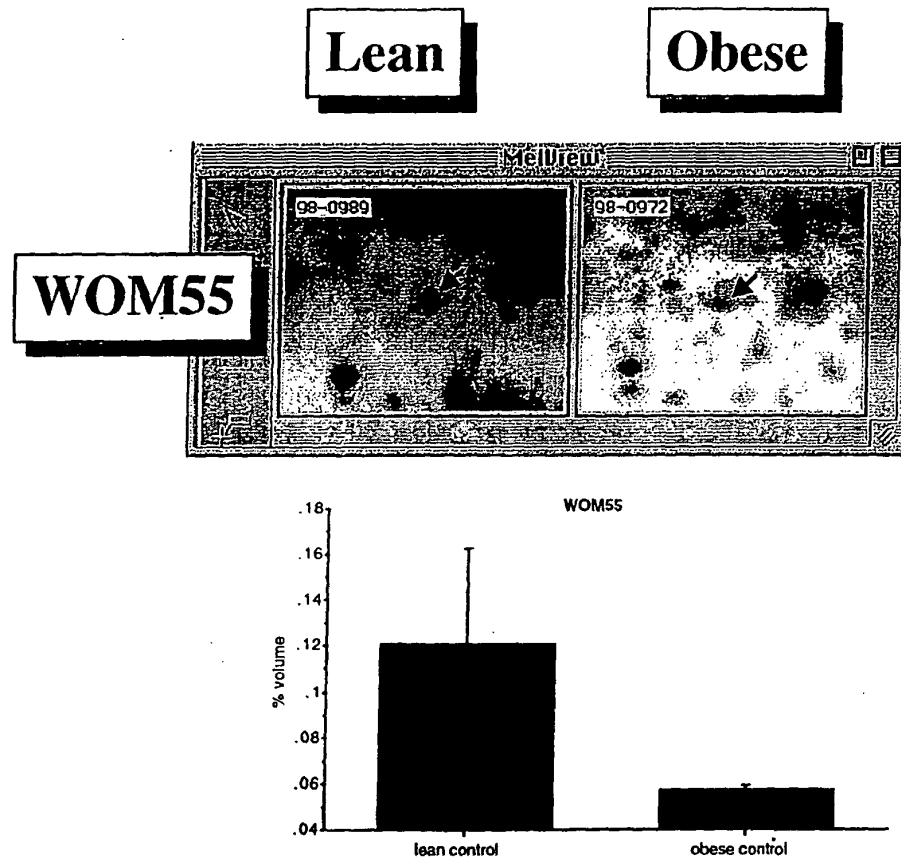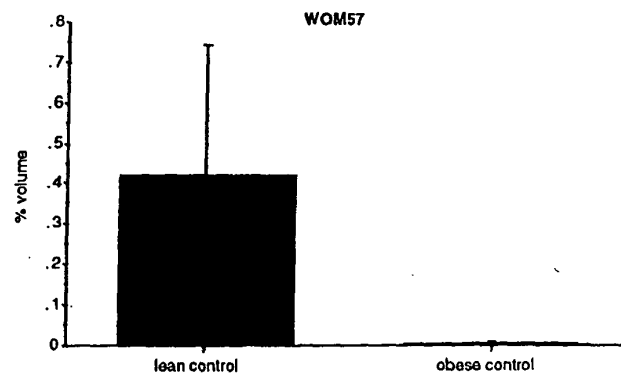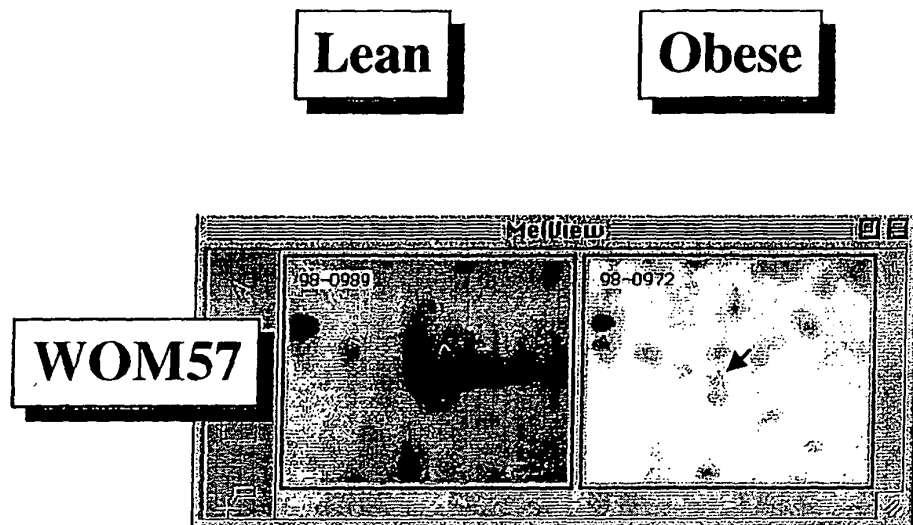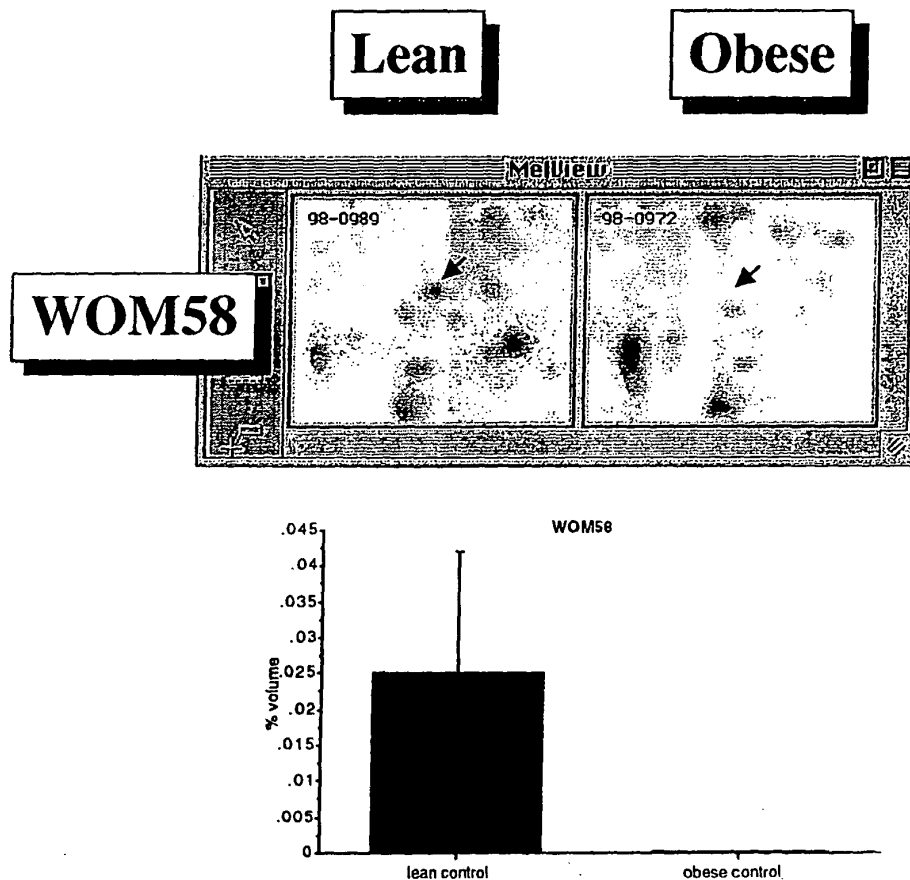
Figure 21

Figure 21 continued

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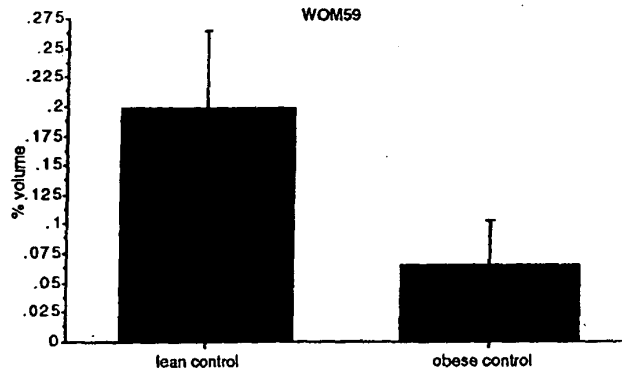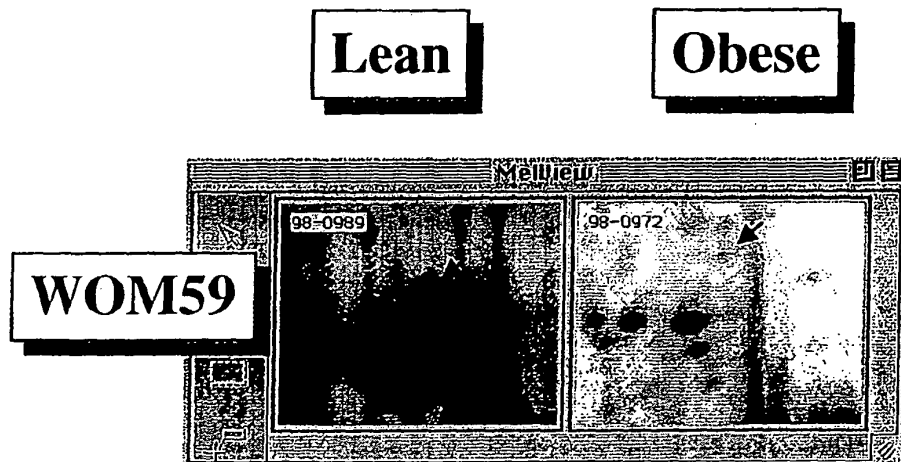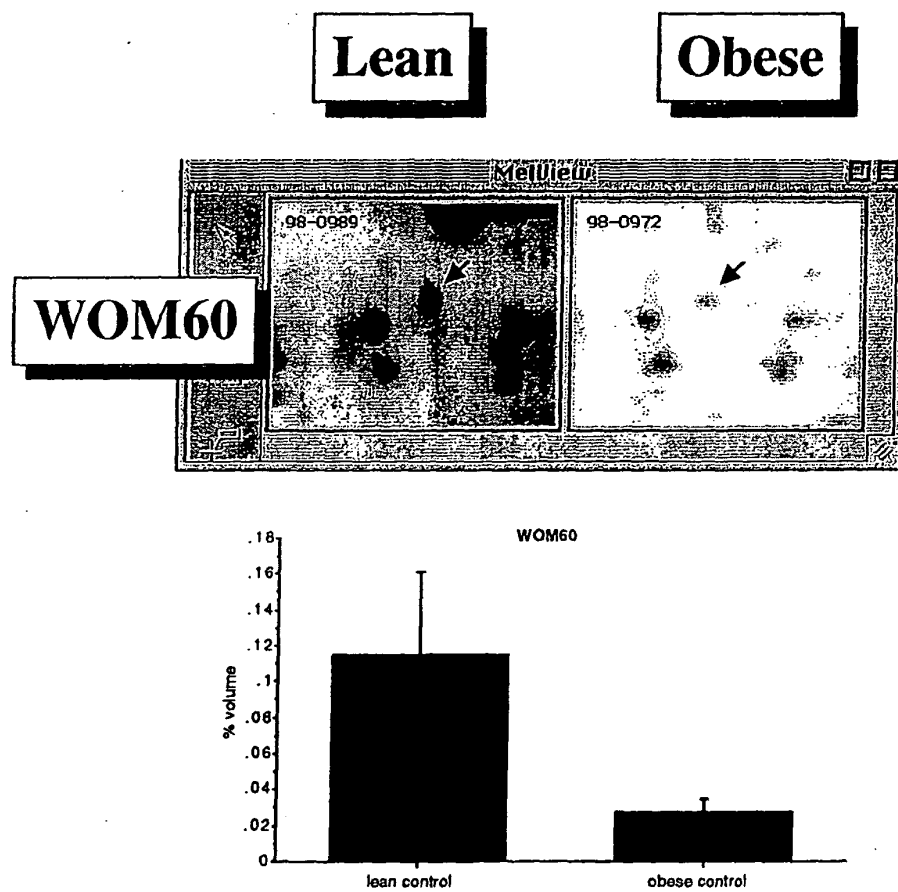
Figure 22

Figure 22 continued

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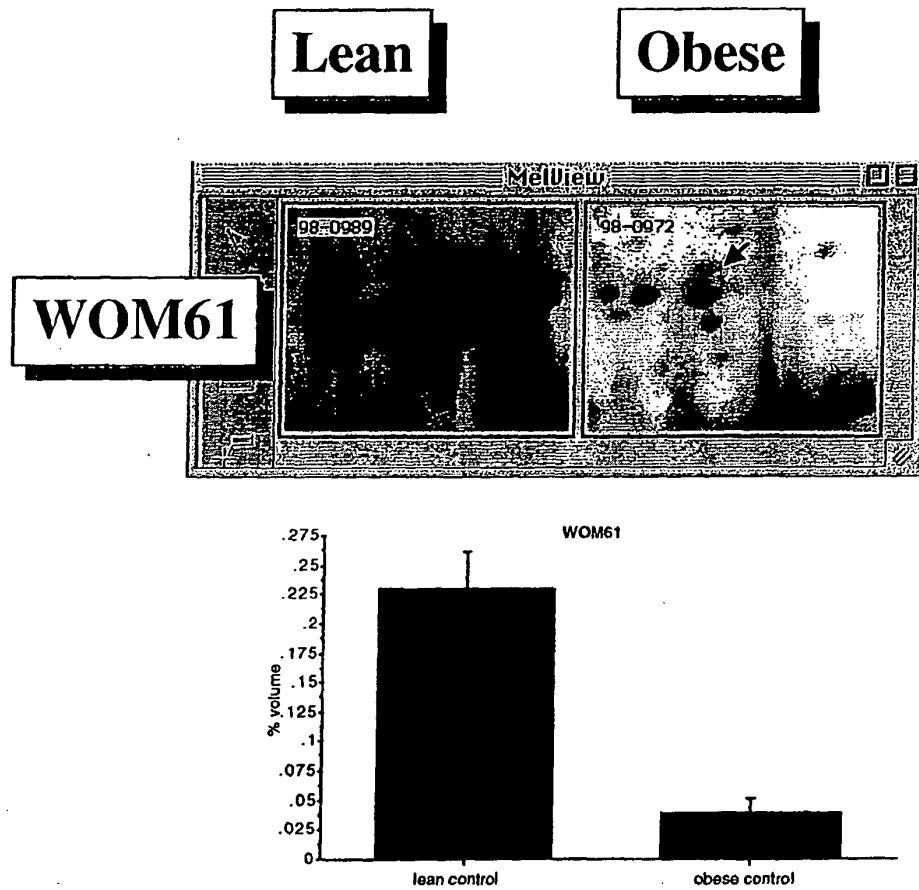
Figure 23

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Figure 23 continued

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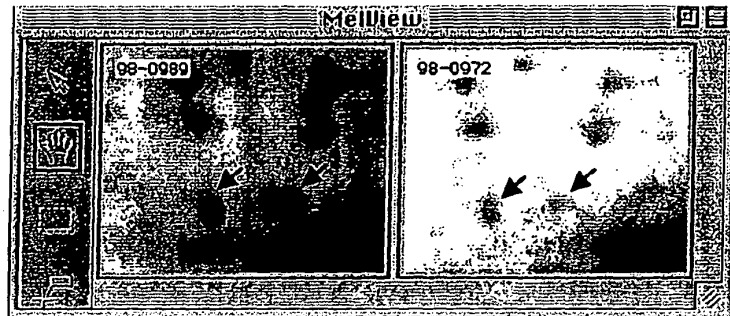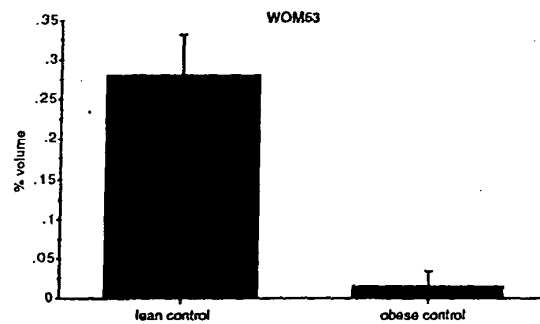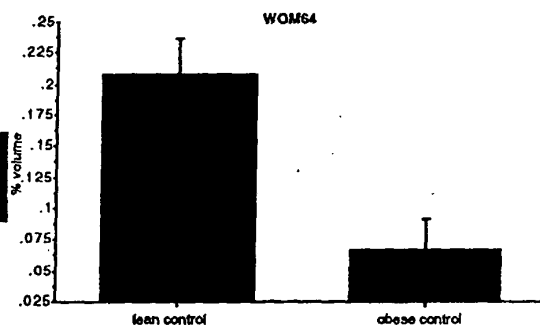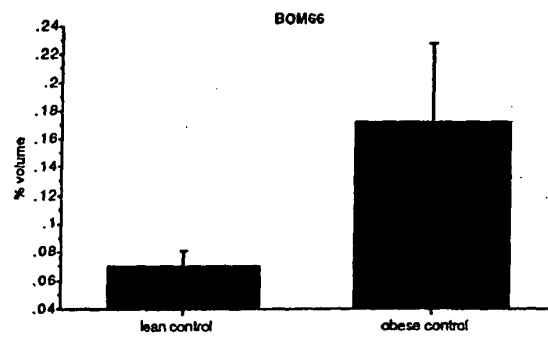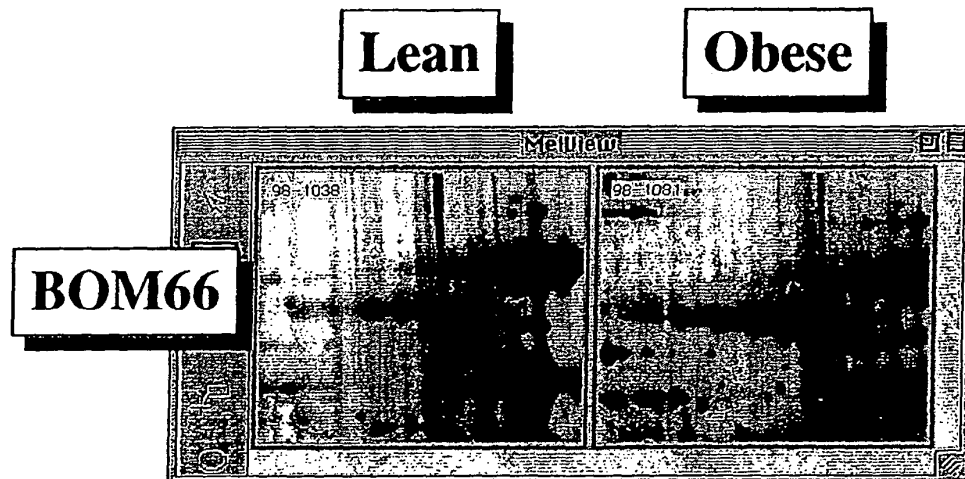
Figure 24

Figure 24 continued

Figure 25

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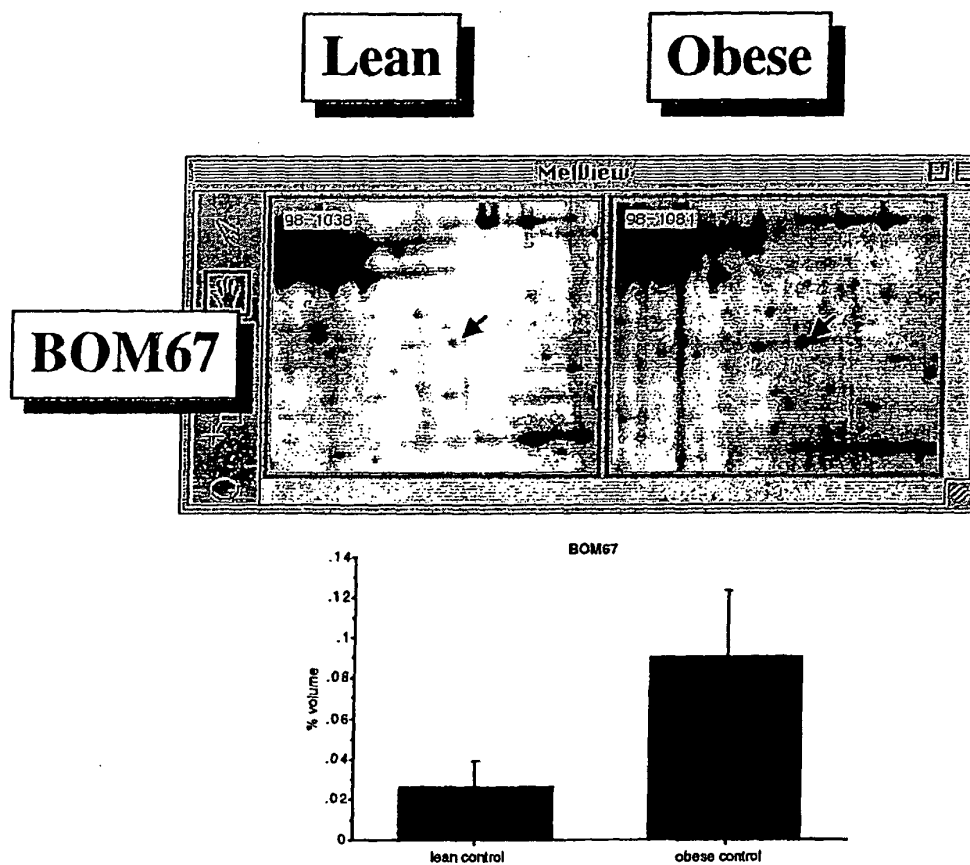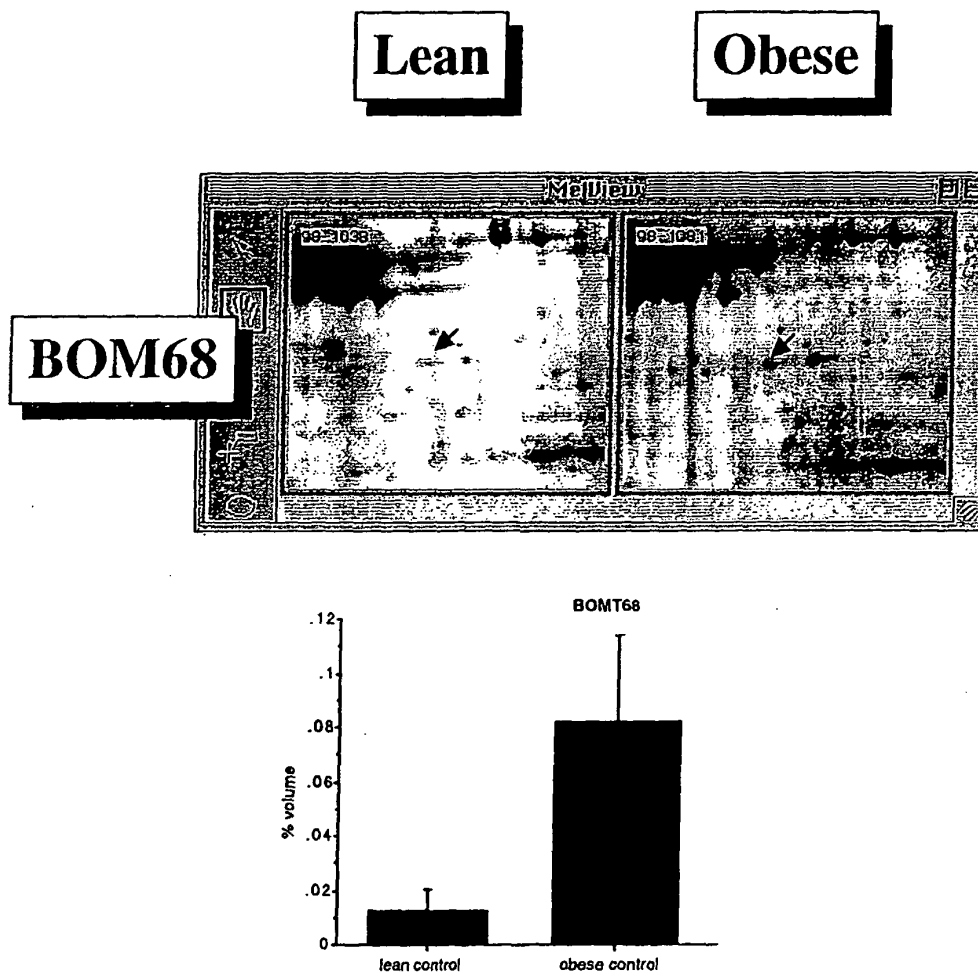
Figure 25 continued

Lean   **Obese**

BOM69

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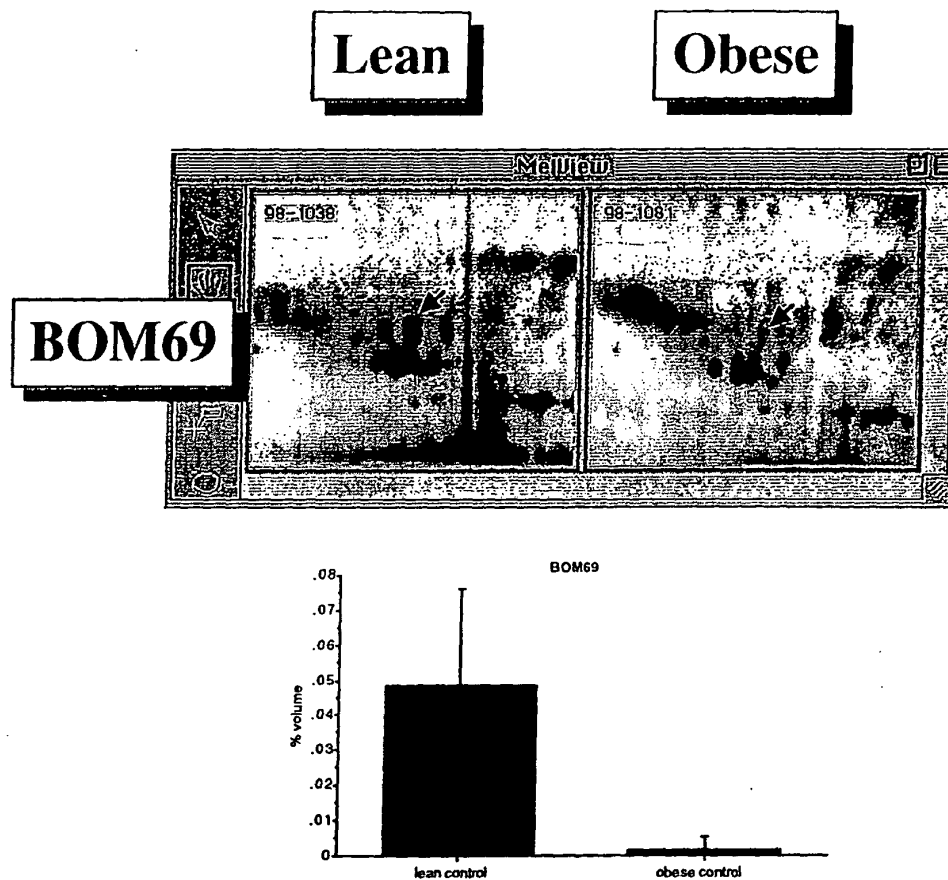
Figure 26
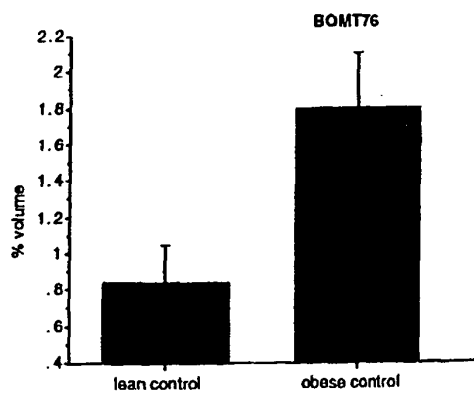
Figure 26 continued

Lean   **Obese**

BOM71

Figure 27

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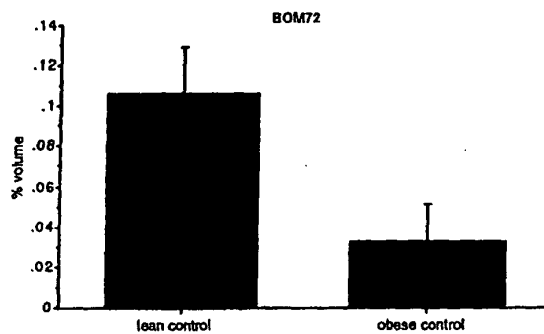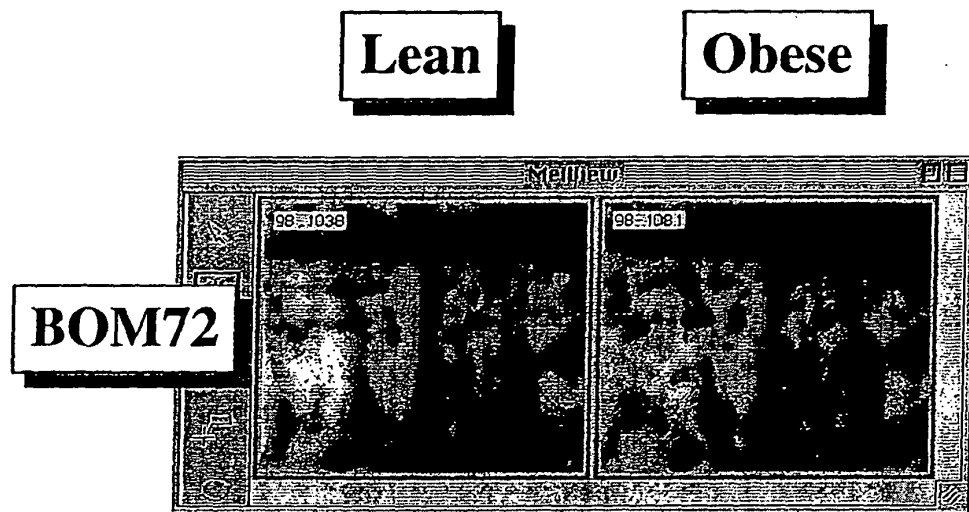
Figure 27 continued

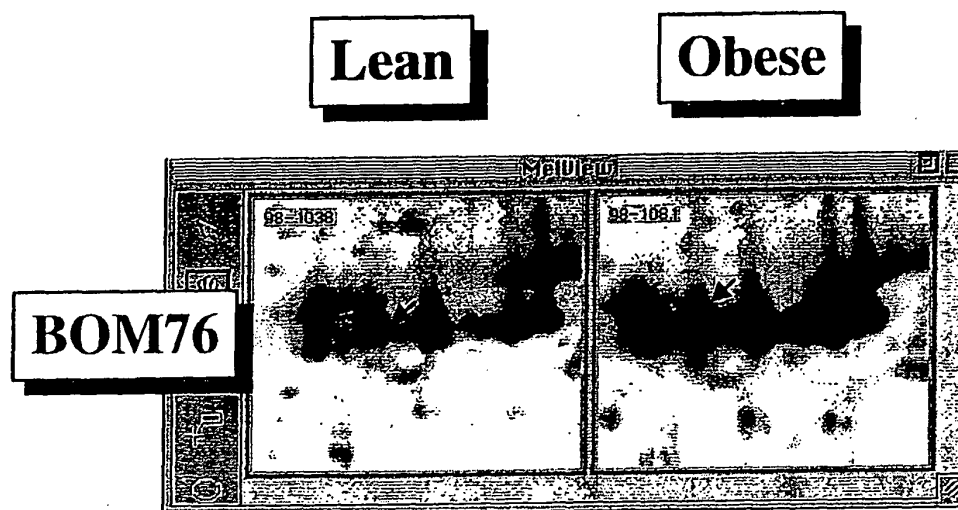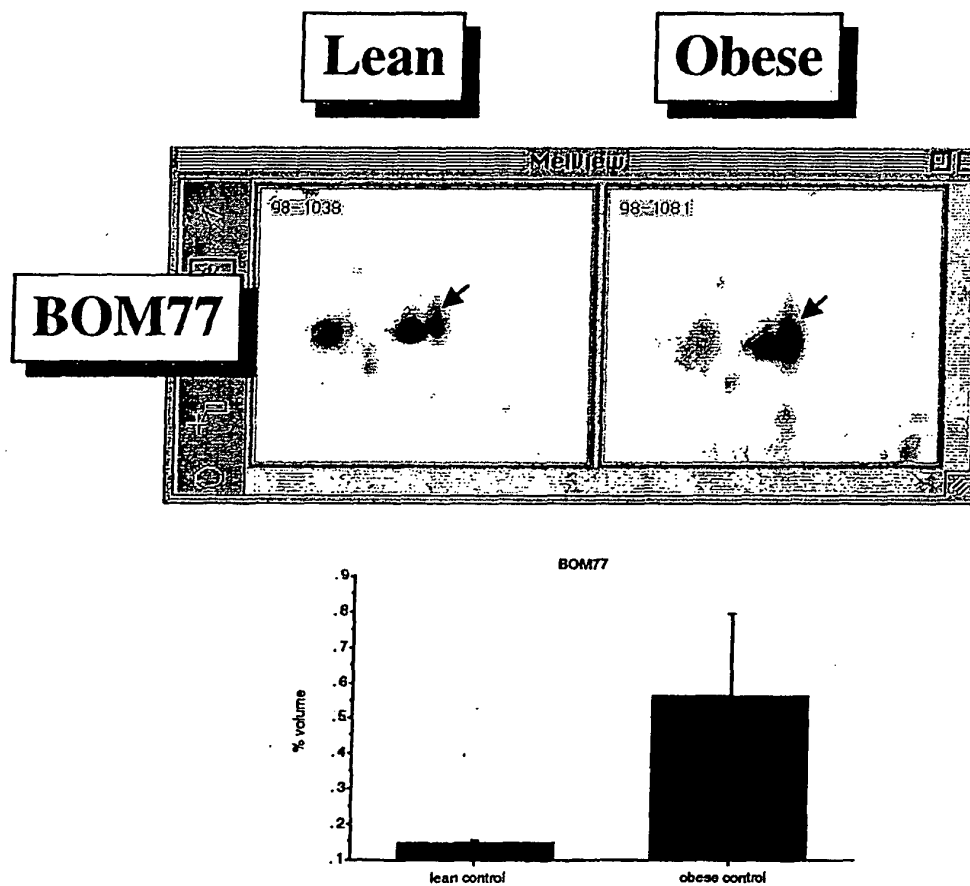**54/58**
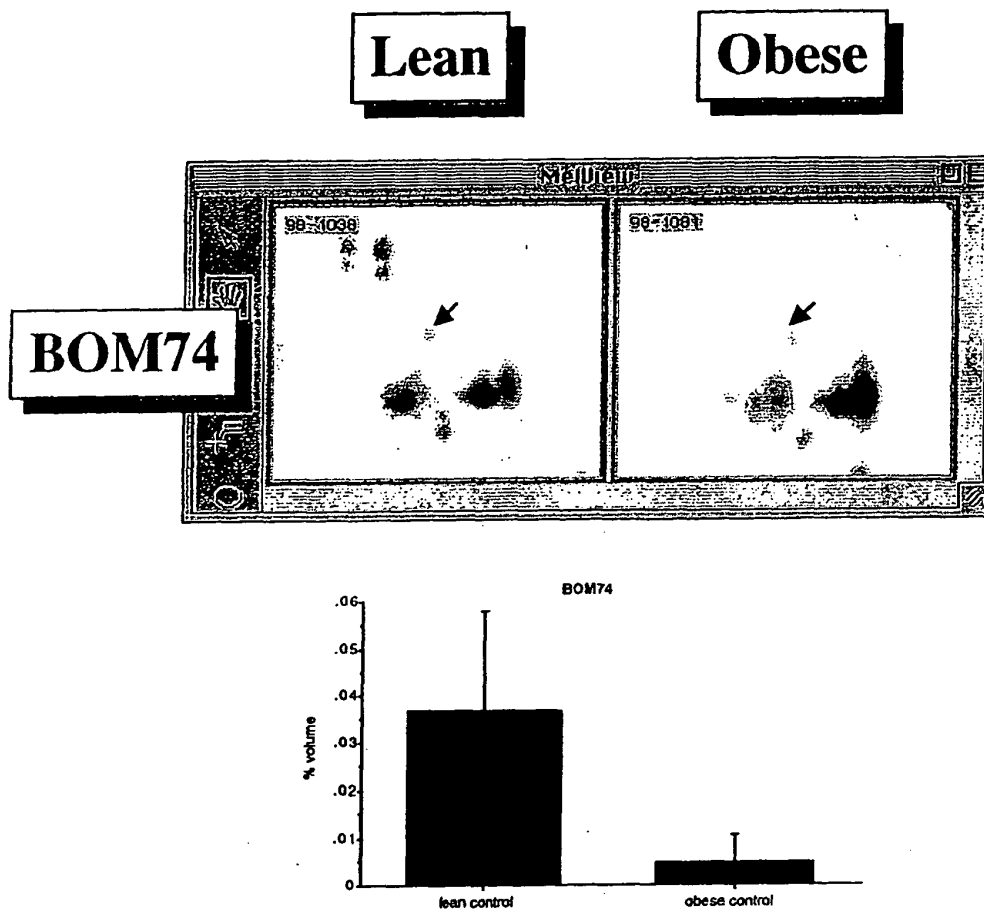
Figure 28

Figure 28 continued

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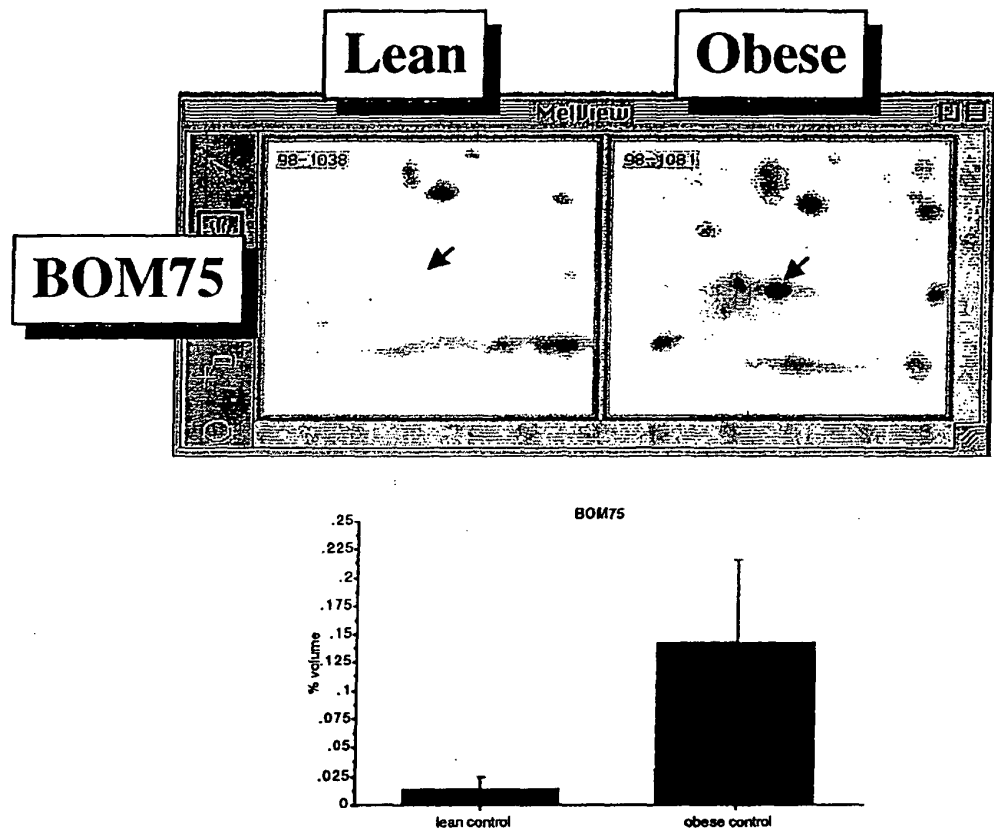
Figure 29

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Figure 29 continued

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